ROLES OF INTERLEUKIN 6, ZINC, AND METALLOTHIONEIN IN CYTOPROTECTION

By

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I dedicate this dissertation to my wife Stephanie in sincere appreciation of her love, patience, and encouragement.

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ABBREVIATION	MEANING
BSA	bovine serum albumin
Cd	cadmium
Ci	Curie
Cu	copper
d	day
δ-ALA-D	δ-aminolevulinic acid dehydratase
EDTA	ethylenediamine tetraacetic acid
FBS	fetal bovine serum
g	gram
h	hour
HSF	hepatocyte stimulating factor
IL-1α	interleukin 1α
IL-6	interleukin 6
ip	intraperitoneal
kg	kilogram
LAF	lymphocyte activating factor
LDH	lactate dehydrogenase
mg	milligram
min	minute
ml	milliliter
MT	metallothionein
mol	mole
ng	nanogram
pI	isoelectric point
pmol	picomole
rhIL-1α	recombinant human IL-1α
rhIL-6	recombinant human IL-6
sec	seconds
SEM	standard error of mean
μCi	microcurie
μg	microgram
μl	microliter
μmole	micromole
Zn	zinc

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The objective of this research was to utilize hepatocytes as a cellular model to study zinc deficiency, regulation of metallothionein synthesis and zinc metabolism by acute-phase mediators, and cytoprotection by inducers of metallothionein synthesis and zinc accumulation. Rat hepatocytes were isolated by collagenase perfusion and cultured in monolayers to address specific research objectives. Hepatocytes were evaluated as a cellular model of zinc deficiency by examining the effects of zinc-deficient culture medium containing 1 μM zinc on a variety of zinc-dependent parameters. Hepatocytes cultured in zinc-deficient medium maintained cell zinc similar to livers of zinc-deficient animals even when high levels of the zinc-binding ligands EDTA and BSA were present in the medium. Similarly, zinc-deficient medium did not affect metallothionein, metallothionein mRNA, de novo protein synthesis, or apparent membrane integrity but reduced activity of the zinc-metalloenzyme δ-aminolevulinic acid dehydratase (δ-ALA-D). In comparison, medium containing 16 or 48 μM zinc restored δ-ALA-D

activity and increased cell zinc and metallothionein expression. Since they maintained cell zinc despite a partial loss of δ-ALA-D activity, hepatocyte monolayers may be a good model to study soft tissue zinc deficiency. Next, hepatocytes were utilized to evaluate the abilities of cytokines to regulate metallothionein expression and zinc metabolism. Interleukin 1α was examined because it increases hepatic metallothionein expression when administered in vivo and interleukin 6 was studied because it induces synthesis of other hepatic acute-phase proteins. Interleukin 6 produced concentration- and time-dependent increases in both metallothionein protein and mRNA while interleukin 1α had no effect. Interleukin 6 also increased cellular zinc concentrations. Each interleukin 6 effect required the glucocorticoid hormone dexamethasone and was optimized by added zinc. Therefore, at the level of the hepatocyte, interleukin 6 rather than interleukin 1α is a major mediator of metallothionein expression and zinc metabolism. To determine whether metallothionein induction and zinc accumulation could provide cytoprotection, hepatocytes were pre-treated with combinations of zinc, dexamethasone, and interleukin 6 to induce metallothionein and then were exposed to cytotoxic compounds. Carbon tetrachloride-induced cell death and lactate dehydrogenase leakage were reduced in hepatocytes in which metallothionein and zinc accumulation were previously induced. Thus, interleukin 6 provided cytoprotection via a mode consistent with dependence upon increased cellular metallothionein and/or zinc.

INTRODUCTION

Metallothionein is a cysteine-rich, metal-binding protein that is intimately involved in the metabolism of zinc. Not only does zinc bind to and stabilize apometallothionein (Dunn et al., 1987), but dietary zinc also transcriptionally regulates metallothionein expression (Blalock et al., 1988). In addition, metallothionein synthesis is induced by glucocorticoids and stress-related mediators with interleukin 1-like activity (Cousins, 1986). Metallothionein induction triggered by interleukin 1 causes a transient depression of zinc in the plasma and concomitant uptake of zinc by the liver, bone marrow, and thymus (Cousins and Leinart, 1988; Huber and Cousins, 1988). The mechanisms by which these changes are regulated at the cellular level are not known.

Based on metallothionein's purported ability to scavenge hydroxyl radicals (Thornalley and Vasak, 1985) and zinc's stabilizing effect on cell membranes (Bettger and O'Dell, 1981; Girotti et al., 1986), cytoprotective functions for metallothionein and zinc have been postulated (Thomas et al., 1987; Coppen et al., 1988; Abel and Ruiter, 1989). There is a need to better understand these functions.

In the present study, monolayer cultures of adult rat hepatocytes were used as a cellular model to examine zinc deficiency, regulation of metallothionein expression and zinc metabolism by the cytokines interleukin 1 and interleukin 6, and cytoprotection by inducers of metallothionein synthesis.

REVIEW OF LITERATURE

Zinc

Essentiality

Although Raulin first recognized the necessity of zinc for growth of microorganisms in 1869 (Raulin, 1869), the essentiality of zinc in animals was not demonstrated until 1934 by Todd and coworkers (1934). They observed that rats fed zinc deficient but otherwise nutritionally complete diets grew poorly and lost body hair. Later Tucker and Salmon (1955) demonstrated zinc deficiency in pigs and O'Dell and colleagues (1958) showed zinc deficiency in chickens. Zinc deficiency in humans was first documented in the early 1960s by Prasad and coworkers in the Middle East (Prasad et al., 1961; 1963).

Human zinc deficiency is most common in developing countries in which cereal and leguminous foods make up the bulk of the staple diet (Solomons and Cousins, 1984). The lower availability of zinc from these foods as compared to meats has been attributed to the presence of inhibitors of zinc absorption such as phytate (Reinhold, 1971; Reinhold et al., 1973) and fiber (Pecoud et al., 1975). The possibility of zinc deficiency in developed countries was emphasized by Hambidge and coworkers (1972). They found that low zinc concentration in the hair of school children living in Denver, Colorado, correlated with poor growth and anorexia.

Symptoms of nutritional zinc deficiency include anorexia, growth retardation, alopecia, dermatitis, reproductive dysfunction, skeletal defects, mental lethargy, hypogeusia, and impaired immunocompetence (Prasad et al., 1961; 1963; Mahajan et al., 1980; Fraker et al., 1982). These symptoms have been reviewed in detail (Hambidge et al., 1986).

The biochemical bases for the symptoms of nutritional zinc deficiency are not understood. The most notable biochemical change in zinc deficient animals is decreased plasma zinc concentration. Some deficiency symptoms are probably the result of altered enzyme activity since zinc functions as a component of many zinc-metalloenzymes (Vallee and Galdes, 1984). However, other functions of zinc are probably also involved since the zinc concentrations of most soft tissues and the activities of many zinc-metalloenzymes are not significantly altered, even in severely zinc deficient animals (Kirchgessner et al., 1976). In addition, many symptoms and physiological changes associated with nutritional zinc deficiency are manifested more rapidly than changes in zinc metalloenzyme activities (Bettger and O'Dell, 1981). These facts stress the importance of further research regarding the cellular metabolism of zinc.

Chemistry

Zinc is a first series transition metal. All transition elements of the first series have an inner argon core surrounded by varying numbers of valence electrons located in the 4s and 3d subshells. Zinc is located at the end of the series with the electronic configuration $[Ar]4s^2 3d^{10}$.

Since valence 3d electrons are of nearly equal energy and because they are readily removed, most first series transition metals normally exist in nature in more than one oxidation state (Phillips and Williams, 1966). However because of its d^{10} configuration, zinc prevails in the +2 oxidation state. The Zn^{2+} ion does not share many of the common characteristics of most transition elements (Douglas et al., 1983). For example, Zn^{2+} is diamagnetic rather than paramagnetic because all of its electrons are paired. Also Zn^{2+} will not form colored compounds since all of its subshells are filled and light can not shift an electron from one energy level to another.

One characteristic that zinc shares with other transition elements is the ability to bind ligands noncovalently to form coordination complexes. This property is biologically important because ligands dictate the absorbability of zinc, facilitate the transport and storage of zinc, and impart to zinc the ability to perform vital biochemical and physiological functions. The arrangement of ligands about a zinc nucleus depends on the ligands involved. The most common arrangements are the 4-coordinate tetrahedral and 6-coordinate octahedral geometries (Nebergall et al., 1976).

The affinity of a ligand for a metal ion such as Zn^{2+} is most easily understood when considered in terms of an acid-base interaction. Due to its high charge density, Zn^{2+} can act as a Lewis acid to withdraw electrons from electron-rich functional groups of ligands. Physiologically ubiquitous ligands which bind zinc tightly include aspartate, glutamate, lysine, histidine, tyrosine, cysteine, arginine, and N-terminal amino acids (Hughes, 1984). These amino acids contain

nitrogen, sulfur, and oxygen which are good Lewis bases (Basolo and Pearson, 1967).

When several different ligands are present, competitive equilibria result and a variety of zinc complexes are formed. The formation of zinc complexes is also affected by a number of physiochemical factors which are beyond the scope of this review. A combination of all of these factors forms the basis for the biochemical and physiological functions of zinc.

Functions

Zinc metalloenzymes

As noted, zinc is a component of many metalloenzymes (Vallee and Galdes, 1984). Carbonic anhydrase was the first zinc metalloenzyme to be identified (Keilin and Mann, 1940). Today the list of known zinc metalloenzymes contains members of all six enzyme classes and includes RNA nucleotidyl transferases, alcohol dehydrogenase, alkaline phosphatase, and δ-aminolevulinic acid dehydratase (Vallee and Galdes, 1984). In these enzymes zinc provides structural integrity and/or participates in catalysis. Zinc's involvement in a wide variety of metabolic pathways via zinc metalloenzymes probably accounts for some of the physiological symptoms associated with nutritional zinc deficiency.

Zinc fingers

Recently a novel DNA-binding motif was discovered by investigators who were studying the amino acid sequence of Xenopus transcription factor IIIA (Miller et al., 1985). The researchers recognized small units repeated in tandem and subsequently proposed that each unit folds around a Zn^{2+} ion to form individual structural domains. This motif,

referred to as a "zinc finger," is ubiquitous since other investigators have found similar units in the amino acid sequences of other transcription factors and viral proteins (Sunderman and Barber, 1988).

Several subclasses of zinc fingers have now been identified (Sunderman and Barber, 1988). The classical arrangement of transcription factor IIIA has a Zn^{2+} ion coordinated to a doublet of cysteine and a doublet of histidine in a tetrahedral arrangement. This configuration has inner ligands separated by 12 or 13 amino acids. In addition, at least two other subclasses have been found. The most common features a sequence with the same spacing but contains two doublets of cystiene residues. Less frequently a histidine doublet separated by four or five amino acids from a cysteine doublet is observed.

Zinc finger motifs may impart strength and specificity of DNA-binding to regulatory proteins (Sunderman and Barber, 1988). Specificity could be accomplished by varying the number of amino acids in each domain, the number of amino acids between domains, and the number of zinc fingers themselves. Strength of binding could be modulated by varying the amino acid sequence of each domain.

Presently, the impact of nutritional zinc deficiency on zinc fingers can only be hypothesized. Since zinc appears to play a structural role in these transcription factors, improper folding may occur in the absence of zinc. As a result, the sensitivity of gene expression to hormones like glucocorticoids (Freedman et al., 1988), would be reduced in zinc deficiency and cell dysfunction and concomitant pathology could develop.

Cytoprotective role

A rapidly growing line of research suggests that in addition to its role as a component of zinc metalloenzymes and zinc fingers, zinc also exerts an essential cytoprotective role by stabilizing membrane structure (Bettger and O'Dell, 1981). This role for zinc is supported by the observation that lipid peroxidation is elevated in some tissues of zinc deficient animals (Sullivan et al., 1980). Such a role could explain the development of skin disorders as well as other symptoms characteristic of zinc deficiency.

Bettger and O'Dell (1981) have suggested that this cytoprotective function is the result of a direct effect of zinc on the cell membrane. This hypothesis is based, in part, on studies which used erythrocytes (Bettger et al., 1978; Bettger and Taylor, 1986; Chvapil et al., 1974). Erythrocytes from zinc deficient rats exhibited increased osmotic fragility compared to controls while erythrocytes from zinc adequate rats exhibited decreased osmotic fragility when zinc was added. The increased osmotic fragility of the erythrocytes from zinc deficient rats was postulated to be a function of zinc concentration since the membranes contained significantly less zinc than controls.

The hypothesis that zinc exerts a direct cytoprotective effect on cell membranes was supported by Girotti and coworkers (1986). They showed that the addition of Zn^{2+} to erythrocyte ghosts concurrently with xanthine, xanthine oxidase, and Fe^{3+} significantly decreased H_2O_2 and O_2^- dependent lipid peroxidation compared to controls which did not receive added Zn^{2+}. Since xanthine oxidase activity was not affected, the

investigators concluded that Zn^{2+} uptake by the membranes was responsible for the cytoprotective effect.

Ludwig and Chvapil (1982) suggested that zinc may also stabilize membranes indirectly. They hypothesized that zinc inhibits carbon tetrachloride toxicity by stabilizing NADPH which, in turn, can inhibit the microsomal drug oxidizing system which metabolizes carbon tetrachloride to harmful $CCl_3^{\cdot}$ radicals. Through propagation reactions with other molecules, $CCl_3^{\cdot}$ radicals can generate other radicals which may cause membrane damage and cell death via peroxidation of polyunsaturated fatty acids. Alternatively, an indirect cytoprotective effect of zinc could potentially be mediated via the synthesis of the zinc-binding protein metallothionein (Thornalley and Vasak, 1985).

Metallothioneins

The term metallothionein actually refers to a family of proteins found ubiquitously in eukaryotic species. A unique property of metallothioneins is that they bind stoichiometric quantities of heavy metals such as cadmium, copper, and zinc. This property has led investigators to hypothesize roles for metallothionein in regulating heavy metal detoxification and essential metal homeostasis. Detailed reviews of metallothioneins are available (Kagi and Nordberg, 1979; Hamer, 1986; Dunn et al., 1987).

Physiochemistry

All metallothioneins are single-chain polypeptides of 60 to 61 amino acids with N-acetylmethionine and alanine at the amino and carboxyl termini, respectively (Kagi and Nordberg, 1979). The primary structure of metallothioneins is characterized by a high content of

cysteine residues ranging from 23 to 33 percent with no disulfide bonds, histidines, or aromatic amino acids (Dunn et al., 1987). The distribution of cysteinyl residues along the peptide chain is highly conserved due to their involvement in binding metals.

Another distinctive characteristic of metallothioneins is their low molecular weight (Dunn et al., 1987). Sequence data of mammalian metallothioneins indicate a molecular weight for the native protein of about 6000. Depending upon the metal composition, the actual molecular weight can range from 6500 to 7000. X-ray crystallography has shown that metallothioneins have an ellipsoid shape (Furey et al., 1986). This asymmetry explains the apparent molecular weight of 10,000 usually determined by gel filtration chromatography.

Metallothioneins contain two globular domains (Furey et al., 1986). The domain within the carboxyl terminal end from amino acid 31 through 61 is referred to as "α" while the domain that spans the amino terminal end from residue 1 through 30 is called "ß". The "ß" domain contains nine cysteine residues and binds either three Zn^{2+} or Cd^{2+} ions, or six Cu^{1+} ions (Nielson et al., 1985). The "α" domain contains eleven cysteine residues and binds either four Zn^{2+} or Cd^{2+} ions, or five Cu^{1+} ions. The relative affinities of metallothioneins for these metals are $Cu^{1+}>Cd^{2+}>Zn^{2+}$, however Zn^{2+} is the most physiologically ubiquitous (Hamer, 1986). Each Zn^{2+} ion is bound to metallothioneins by four cysteine thiolate ligands in a tetrahedral coordination complex (Hamer, 1986).

Metal binding reduces metallothionein degradation and increases stability of the protein (Dunn et al., 1987). Thus the intracellular

concentration of metallothioneins is dependent upon zinc. When dietary zinc is extremely low, intracellular levels of zinc are reduced and metallothioneins in some tissues are degraded (Cousins, 1985).

The relative rates of turnover of metallothioneins with differing metal compositions are not well understood. Zn-metallothioneins and Cd-metallothioneins are resistant to degradation by selected cytosolic proteases but susceptible to lysosomal breakdown (Feldman et al., 1978). In contrast, Cu-metallothioneins are resistant to both cytosolic and lysosomal degradation (Held and Hoekstra, 1979). A better understanding of these mechanisms may provide insight into the functions of metallothioneins.

Regulation by Metals

Metals that bind to metallothioneins also transcriptionally activate metallothionein genes when the metals reach threshold levels within cells. This has been demonstrated by administering metal salts to intact animals by injection (Feldman et al., 1978) or feeding (Blalock et al., 1988), or to cultured cells via the culture medium (Failla and Cousins, 1978a). The relative abilities of metals to induce metallothioneins are $Cd^{2+}>Zn^{2+}>Cu^{1+}$.

Metals induce metallothionein expression by interacting with metal regulatory elements in promoter regions located upstream from the metallothionein structural genes (Hamer, 1986). The mechanism of this interaction may involve metal binding to a nuclear regulatory protein which in turn binds to the DNA sequence of the metal regulatory element (Cousins et al., 1988).

Regulation by Hormones

Metallothionein synthesis is also up-regulated by hormones associated with acute trauma including glucocorticoids, glucagon, epinephrine, dibutyryl cAMP, endotoxins, and interleukin 1 (Cousins et al., 1986). Interleukin 1 produces a tissue-specific redistribution of zinc with a transient depression of zinc in the plasma and concomitant uptake of zinc by the liver, bone marrow, and thymus (Cousins and Leinart, 1988). Models constructed via the SAAM and CONSAM modeling programs mathematically describe the relationship between these hormonally-induced changes and metallothionein induction (Dunn and Cousins, 1989). Thus, redistribution of body zinc stores by tissue-specific hormonal regulation of metallothionein synthesis appears to be an important part of the body's acute response to stress.

Several mediators of metallothionein synthesis probably act directly at the level of the hepatocyte. A putative regulatory element for glucocorticoid hormones in the promoter region of metallothionein genes has been identified (Hamer, 1986). In addition, incubation of hepatocytes with glucocorticoid hormones increases both metallothionein concentration and zinc uptake (Failla and Cousins, 1978a; 1978b). Glucagon and epinephrine also increase metallothionein concentration and zinc uptake in cultured hepatocytes (Cousins and Coppen, 1987). The effects of these polypeptide hormones may be mediated intracellularly via cAMP since metallothionein genes contain potential cAMP regulatory elements (Nebes et al., 1988). Also some studies suggest cAMP induces metallothionein expression in cultured cells (Cousins and Coppen, 1987;

Nebes et al., 1988) however others indicate cAMP has no effect (Nebes et al., 1990).

Endotoxin does not have a direct effect on metallothionein induction in primary hepatocytes. Instead, the effect of endotoxin may be mediated in some way via the cytokine interleukin 1. Interleukin 1 is a low molecular weight protein that is released from activated macrophages, monocytes, and other cell-types in acute response to infection as well as other trauma such as tissue injury and stress (Dinarello, 1988). Once released interleukin 1 triggers the up-regulation of a broad spectrum of systemic acute-phase responses involved in host defense.

The mechanism by which interleukin 1 regulates metallothionein gene expression and zinc metabolism in hepatocytes is not clear. A direct action at the cellular level could involve multiple modes of signal transduction (Dunn and Cousins, 1989; Nebes et al., 1988; Imbra and Karen, 1987). In addition, interleukin 1 stimulates glucocorticoid release via its corticotropin-releasing activity on pituitary cells (Woloski et al., 1985). Therefore, interleukin 1 could affect metallothionein synthesis, in part, via glucocorticoid hormones.

Other cytokines such as interleukin 6 (also referred to as interferon ß2 and hepatocyte stimulating factor), could mediate interleukin 1 effects at the level of the hepatocyte. Like interleukin 1, interleukin 6 is a low molecular weight cytokine which is released from a variety of cell-types including monocytes (Dinarello, 1988). Interleukin 1 stimulates interleukin 6 synthesis in some cells (Van Damme et al., 1987; Walther et al., 1988; Zhang et al., 1988).

Furthermore, interleukin 6 regulates the synthesis and secretion of a variety of acute-phase hepatic proteins (Andus et al., 1988) and synergizes with interleukin 3 to increase proliferation of hematopoietic progenitor cells (Ikebuchi et al., 1987).

Functions

Homeostatic roles

A variety of homeostatic roles have been proposed for metallothioneins. Metallothioneins may regulate zinc homeostasis by functioning as a mucosal block to zinc absorption (Cousins, 1979) similar to the original regulatory role proposed for ferritin in iron absorption (Granick, 1946). Metallothioneins may also function in ligand-exchange reactions as zinc donors for activation of metalloenzymes and "zinc finger" motifs of DNA-binding transcription factors (Otvos et al., 1989; Cousins and Hempe, 1990). Metallothioneins could mediate zinc's effects on cellular processes analogous to the way calmodulin mediates calcium's effects (Cheung, 1982).

Cytoprotective roles

Several cytoprotective roles for metallothioneins have been proposed. First, because they are induced by and form complexes with metals such as cadmium and copper, metallothioneins provide protection from heavy metal toxicity (Hamer, 1986). The success of oral zinc therapy in preventing copper toxicity in Wilson's disease patients probably is due to sequestration of hepatic copper in metallothionein induced by zinc (Lee et al., 1989). Studies utilizing cells transfected with metallothionein genes indicate metallothioneins also provide cytoprotection against alkylating agents (Kelley et al., 1988). In

addition, in vitro data suggest the zinc-thiolate clusters in metallothioneins efficiently scavenge hydroxyl radicals (Thornalley and Vasak, 1985). Therefore, metallothioneins may also function in the free radical defense system as hydroxyl radical-scavengers.

Since tissue metallothionein concentrations are dependent upon levels of dietary zinc (Blalock et al., 1988), the hydroxyl radical-scavenger function can be placed within the context of zinc deficiency. In animals with adequate zinc status, the basal level of metallothionein expression may be sufficient to scavenge hydroxyl radicals and prevent lipid peroxidation. However, in zinc deficient animals, reduced levels of metallothioneins could account for increased lipid peroxidation observed in some tissues (Sullivan et al., 1980).

The hydroxyl radical-scavenger role of metallothioneins can also be placed within the context of events which occur during the body's response to infection (Karin, 1985; Cousins, 1985). Following invasion by bacteria or viruses, interleukin 1 is released from a variety of cell-types (Dinarello, 1988). Interleukin 1 activates neutrophils and macrophages leading to the release of large quantities of active oxygen species including superoxide and hydroxyl radicals. These compounds function to destroy the invading bacteria or virus. In the absence of adequate cytoprotective mechanisms, these compounds could cause severe damage to the host. Induction of superoxide dismutase by cytokines provides one form of cytoprotection for cells (Wong and Goeddel, 1988). Superoxide dismutase deactivates superoxide radicals. Induction of metallothioneins triggered by interleukin 1 with concomitant movement of

zinc out of the plasma and into the liver and other specific tissues may provide additional cytoprotection by deactivating hydroxyl radicals.

Recent support for the hydroxyl radical-scavenger hypothesis was provided by a study that assessed the protective effect of zinc against carbon tetrachloride hepatotoxicity in rats (Clarke and Lui, 1986). Zinc (10 mg/kg) or saline was administered by intraperitoneal injection 24 and 2 hours before carbon tetrachloride administration (1 ml/kg, ip). The rats were sacrificed four hours later and liver toxicity was assessed by a variety of indices including serum aspartate aminotransferase activity. Zinc afforded limited protection prior to induction of metallothionein synthesis. Further, chromatographic study of hepatic cytosols showed that metallothionein-bound zinc was selectively depleted by carbon tetrachloride exposure.

Additional support for the radical-scavenger hypothesis was provided by studies in which primary cultures of rat hepatocytes were used to test the influence of zinc on free radical formation and lipid peroxidation (Coppen et al., 1988). Peroxidation was induced in cultures incubated with 1, 16, 24, 32, or 48 μM zinc by either 3-methylindole, tert-butyl hydroperoxide, or iron (II)-nitrilotriacetic acid. Free radical formation was assessed by examination of electron spin resonance spectra of appropriate spin trapped adducts and lipid peroxidation was determined based on malondialdehyde production. Results indicated that free radical formation and lipid peroxidation were significantly reduced in cultures incubated with increasing amounts of zinc where high levels of metallothionein were produced.

At least one inconsistency suggests the hydroxyl radical-scavenger hypothesis is not valid. The bimolecular rate constant reported for reaction of metallothionein with hydroxyl radicals (Thornalley and Vasak, 1985) is one-to-two orders of magnitude greater than the constant's diffusion-controlled, theoretical limit (Cantor and Schimmel, 1980). Therefore the bimolecular rate constant is probably incorrect. Despite this inconsistency, the biological evidence is consistent with a role of metallothionein in cytoprotection.

Hepatocyte-Stimulating Cytokines

The body responds to tissue injury, stress, and infection with a series of local and systemic acute-phase reactions which arrest the injury process, protect against further injury, and initiate repair processes (Gauldie et al., 1989). Local responses include release of arachidonate metabolites and vasoactive amines that alter vascular permeability. Systemic responses include activation of phagocytic cells, continued generation of arachidonate metabolites, and release of many hormone-like polypeptides known as cytokines from various cell-types. Cytokines stimulate proliferation of T and B lymphocytes, promote development of cytotoxic T cells and antibody-producing plasma cells, and activate macrophages and other inflammatory cells (Mizel, 1989). Cytokines also initiate changes in intermediary metabolism including anorexia, fever, gluconeogenesis, glucose oxidation, decreased fatty acid uptake by adipocytes, and increased hepatic synthesis of fatty acids and acute-phase proteins (Klasing, 1988). Most hepatic acute-phase proteins function as either antiproteinases, opsonins, blood-clotting or wound-healing factors, or metal-binding ligands

(Gauldie et al., 1989). The principal hepatocyte-stimulating cytokines responsible for inducing acute-phase protein synthesis are interleukin 1, tumor necrosis factor α, and interleukin 6.

Interleukin 1 and Tumor Necrosis Factor α

Interleukin 1 refers to a family of proteins released from macrophages, monocytes, and other cell-types following stimulation by phagocytosis or by immune complexes (Mizel, 1989). Two forms, interleukin 1α and interleukin 1ß, have been characterized in humans. Each is the product of a different gene and is synthesized as a 33,000 molecular weight precursor which is processed to a 13,000-17,000 molecular weight mature form (Giri et al., 1985). The α and ß forms of interleukin 1 are most easily distinguished physiochemically by their isoelectric points which are 5.0 and 7.0, respectively (Mizel, 1989).

Interleukin 1α and interleukin 1ß bind the same receptor and have the same potencies in bioassays but are differentially secreted (Dinarello, 1988). Interleukin 1α may regulate autocrine events since most of it remains in the cell or associated with the cell membrane. In comparison, much of interleukin 1ß is secreted resulting in circulating levels as high as ten times that of interleukin 1α (Klasing, 1988). Therefore interleukin 1ß may regulate systemic events.

The physiochemical properties of tumor necrosis factor α (TNFα) are similar to those of interleukin 1. In humans, TNFα is synthesized by macrophages and monocytes as a 233 amino acid precurser and is processed to a 17,000 molecular weight mature form with an isoelectric point of 5.3 (Le and Vilcek, 1987; Beutler and Cerami, 1986). Despite these similarities, there is no significant homology between the amino acid

sequences of TNFα and either interleukin 1α or interleukin 1ß and TNFα binds to a different receptor (Klasing, 1988).

Initially, investigators believed that interleukin 1 and TNFα regulated hepatic acute-phase protein synthesis directly since nearly the entire acute-phase response could be mimicked in vivo by administration of either cytokine (Dinarello and Mier, 1987). However, recombinant forms of these cytokines elicit only partial responses in cultured liver cells stimulating synthesis of α_1-acid glycoprotein, hepatoglobulin, and C3 complement in rat hepatoma cells (Baumann et al., 1987) and suppressing synthesis of albumin in rat hepatocytes (Koj et al., 1987). Hence, interleukin 1 and TNFα probably regulate other acute-phase proteins indirectly by stimulating synthesis and release of another cytokine such as interleukin 6.

Interleukin 6

Interleukin 6 synthesis is induced in macrophages, monocytes, fibroblasts, and endothelial cells by interleukin 1, TNFα, and other stimuli (Zhang et al., 1988). Depending upon the cellular source and degree of glycosylation, the molecular weight of interleukin 6 often ranges from 18,000 to 32,000 but molecular weights as high as 70,000 have been reported (Fuller and Grenett, 1989). The larger species may represent precursor forms of interleukin 6. Like interleukin 1 and TNFα, interleukin 6 exerts its biological effects by interacting with a specific saturable receptor.

In contrast to interleukin 1 and TNFα, interleukin 6 regulates a broad spectrum of acute-phase proteins in both rat and human hepatocyte cultures. For human hepatocytes, these proteins include serum amyloid

A, C-reactive protein, hepatoglobulin, α_1-antichymotrypsin, fibrinogen, α_1-antitrypsin, and α_1-acid glycoprotein, as well as the negative acute-phase proteins fibronectin, transferrin, and albumin (Castell et al., 1989). Therefore, interleukin 6 is the primary hepatocyte-stimulating cytokine regulating acute-phase protein synthesis. Since interleukin 1 and TNFα also regulate a subset of acute-phase proteins and because they can synergize or antagonize the effects of interleukin 6, these cytokines probably function as accessory modulators which allow the organism to achieve an effective homeostatic response to the specific tissue injury, stress, or infection that has been encountered.

Hepatocyte Model

The experimental conditions of cell culture can be more closely controlled than conditions of experiments using intact animals (Dagani, 1984). As a result, a variety of cell-types have been utilized to study both zinc deficiency (Flynn and Yen, 1981; Flynn, 1984; Flynn, 1985; Bettger and Taylor, 1986; O'Dell et al., 1987; Falchuk et al., 1975) and acute-phase protein synthesis (Baumann et al., 1987; Koj et al., 1987; Castell et al, 1989).

Zinc Deficiency

Nonproliferating primary hepatocytes, cultured in zinc deficient medium, provide a novel system to study zinc deficiency at the cellular level. Kinetic studies of zinc uptake and exchange have shown that cultured hepatocytes have an average zinc $t_{1/2}$ of 15 hours (Pattison and Cousins, 1986). This suggests that turnover of zinc bound to some cellular ligands is extremely rapid. Also, Guzelian and coworkers (1982) reported that hepatocytes lose up to 70 percent of their zinc and

greater than 90 percent of the activity of the zinc metalloenzyme, δ-aminolevulinic acid dehydratase within 24 hours of culture in zinc deficient medium. These changes parallel those observed in some animal studies in which the hepatic zinc concentration and δ-aminolevulinic acid dehydratase activity were reduced during zinc deficiency (Faraji and Swendseid, 1983). The changes differ from the results of other studies which show that hepatic zinc levels are unaffected by zinc deficiency (Taylor et al., 1988; Murthy et al., 1974; O'Dell et al., 1976). Nevertheless, loss of zinc from specific intracellular pools within hepatocytes could contribute to physiological manifestations of zinc deficiency. The first objective of the present study was to use hepatocytes as a cellular model to study zinc deficiency.

Acute-Phase Zinc Metabolism

Hepatocytes also provide a cellular model to study acute-phase zinc metabolism. Glucocorticoids stimulate metallothionein synthesis and zinc uptake in cultured hepatocytes (Failla and Cousins, 1978a; 1978b). In addition, hepatocytes are the major source of other acute-phase proteins. Consequently, these cells have been used to examine the effects of the cytokine mediators, interleukin 1, tumor necrosis factor α, and interleukin 6 on acute-phase protein synthesis (Castell et al., 1989). The second objective of this study was to utilize hepatocytes as a cellular model to study the regulation of metallothionein synthesis and zinc accumulation by acute-phase mediators. Also cytoprotection by inducers of metallothionein synthesis and zinc accumulation was examined.

MATERIALS AND METHODS

Animals

Male rats (Sprague-Dawley strain; University of Florida Breeding Facility) weighing 150-250g were housed in stainless-steel, suspended cages in a room with a 12 h light-dark cycle (0700 to 1900 h and 1900 to 0700 h, respectively). Rats were fed a standard commercial diet (Purina rat chow, Ralston Purina, St. Louis, MO) and distilled water ad libitum.

Isolation and Maintenance of Rat Hepatocytes

Rats were anesthetized with sodium pentabarbital (65 μg/kg; ip) and hepatocytes were isolated between 0900 and 1100 h by collagenase perfusion (Failla and Cousins, 1978b). Viability of isolated hepatocytes was greater than 85% as judged by trypan blue exclusion.

The fresh hepatocytes were suspended (2.5×10^6 cells/3 ml) in attachment medium which was 10% fetal bovine serum (FBS) and 90% modified Waymouth's MB 752/1 medium (pH 7.4) that contained 15 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid), 10 mM TES (N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid), 30 mM $NaHCO_3$, 0.5 mM sodium pyruvate, streptomycin sulfate (0.1 mg/ml), penicillin G (100 units/ml), gentamycin sulfate (50 μg/ml), alanine (0.41 μM), serine (0.53 μM), and insulin (1 μg/ml). Aliquots (3 ml) were inoculated into 60-mm collagen-coated culture dishes and viable parenchymal cells were allowed to attach selectively over a 3-h period at 37°C.

Following selective attachment, the medium was removed, the cells were washed with a 3 ml aliquot of cell wash buffer (10 mM HEPES, 40 mM NaCl, 7 mM KCl, 1 mg/ml glucose, pH 7.4), and the cells were cultured with fresh, modified Waymouth's MB 752/1 medium supplemented with bovine serum albumin (BSA, 2 mg/ml) and appropriate treatments. For experimental culture periods lasting longer than 24 h, culture media were renewed every 24 h.

Experimental Designs

Zinc Deficiency Study

For zinc deficiency experiments, zinc was extracted from FBS used in the attachment medium so that the zinc concentration of the attachment medium was approximately 1 μM (Table 4). Following attachment, hepatocytes were cultured with BSA-supplemented media containing 1 (the endogenous zinc concentration of BSA-supplemented medium), 16, or 48 μM zinc. These zinc concentrations were chosen to simulate zinc deficient, normal, and repleted plasma, respectively. Supplemental levels of zinc were attained by adding zinc sulfate to the media. After various culture periods, hepatocytes were harvested (Pattison and Cousins, 1986) and appropriate measurements were made.

Acute-Phase Zinc Metabolism Study

For experiments utilizing hepatocytes as a model of acute-phase zinc metabolism, cells were maintained after the attachment period in BSA-supplemented medium for an additional 21 h before medium containing hormones was added. The zinc concentration of the medium was increased from 1 μM to either 16 or 48 μM by adding zinc sulfate. After various culture periods, appropriate determinations were made.

Materials and Analytical Techniques

Extraction of Zinc from Sera and Media

Zinc was extracted from FBS used in the attachment media for zinc deficiency studies via a batch procedure utilizing Chelex-100 resin (Bio-Rad Laboratories, Richmond, CA). Chelex-100 was prepared as described previously (Flynn, 1985) and applied to FBS in a ratio of 1:4 (w/v). After mixing briefly, the resin was removed by centrifugation (5000 x g; 10 min), the pH of the FBS was adjusted to 7.4 with NaOH, and the FBS was sterilized by filtration (0.45 μm Millipore filter). The zinc content of the FBS was then measured by atomic absorption spectrophotometry (Table 4). A similar extraction procedure was utilized to reduce the basal zinc concentration of BSA-supplemented Waymouth's MB 752/1 medium (Table 4) used in one experiment (Figure 2).

Cytokine Preparations

Recombinant human interleukin 1α was provided by Hoffmann-La Roche (Nutley, NJ). Specific activity was $2x10^7$ lymphocyte-activating factor (LAF) units (10^9 D10 units)/mg of protein. Recombinant human interleukin 6 was provided by Genetics Institute (Cambridge, MA). Specific activity was 10^6 hepatocyte-stimulating factor (HSF) units ($7x10^6$ CESS units)/mg of protein.

Measurements

Cytoprotection

Cytoprotection measurements were made using hepatocytes previously treated with zinc and/or hormones. Media were removed and hepatocytes were cultured with BSA-supplemented medium containing iron (II)-nitrilotriacetic acid, tert-butyl hydroperoxide, or carbon

tetrachloride. After appropriate culture periods, relevant measurements were made including malondialdehyde, lactate dehydrogenase leakage, and cell survival (ie. cell protein/dish).

Zinc

For each cellular zinc measurement, hepatocytes from 1-2 dishes were washed twice with EDTA buffer (10 mM HEPES, 150 mM NaCl, 10 mM EDTA, pH 7.4) prior to two washes with cell wash buffer. Then hepatocytes were digested with 0.2% sodium dodecylsulfate in 0.2 N NaOH and zinc was measured by atomic absorption spectrophotometry using a set of zinc standards ranging in concentration from 0.1 to 1.0 ppm. Media and sera zinc concentrations were measured directly by atomic absorption spectrophotometry using similar zinc standards.

Zinc efflux

For zinc efflux measurements, hepatocyte zinc pools were pre-labelled by including $^{65}Zn^{2+}$ (300 nCi/dish) in the attachment medium. After attachment, hepatocytes were washed once with EDTA buffer and once with cell wash buffer. Then BSA-supplemented medium containing appropriate zinc treatments was added. Hepatocytes used for zinc efflux measurements were harvested similarly to those used for total cellular zinc measurements and $^{65}Zn^{2+}$ was measured by liquid scintillation (Beckman LS 7500).

De Novo synthesis of hepatocyte proteins

Hepatocytes previously treated with various levels of zinc were cultured with methionine-free medium containing the same levels of zinc and 50 μCi of ^{35}S-methionine per dish to examine de novo protein synthesis. After 3 h, hepatocytes from 1 dish were harvested in 500 μl

of solubilization solution (9.3 M urea, 5 mM K_2CO_3) and sonicated. Then 25 µl of 4 mM phenylmethylsulfonyl fluoride, 10 µl of Nonidet P-40, and 50 µl of 0.3 M dithiothreitol were added and particulate matter was pelleted by centrifugation (500 x g).

Solubilized hepatocyte proteins were analyzed by two-dimensional polyacrylamide gel elctrophoresis using a modification of the O'Farrell procedure described by Roberts and coworkers (1984). Proteins were separated in the first dimension according to their isoelectric points in a pH gradient (pH 3.5-10) established using a mixture of ampholytes. The tubular isoelectrically focused gel was incubated with equilibration buffer (65 mM Tris, 1 % SDS, 1 % ß-mercaptoethanol, pH 6.8) and affixed atop a 10 % acrylamide slab for protein separation by SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel was stained with Coomassie Brilliant Blue dye and exposed to X-ray film to produce an autoradiogram.

Metallothionein

Metallothionein was measured by the cadmium binding assay (Eaton and Toal, 1982) using hepatocytes from 1-6 dishes. Hepatocytes were harvested in 0.5-1 ml of 10 mM Tris-HCl buffer (pH 7.4), homogenized (Polytron with P10 generator, Brinkman), and centifuged (10,000 x g; 10 min; 4°C). Then the supernatant was heated (100°C; 5 min) and centrifuged (10,000 x g; 5 min). Next, 200 µl of cadmium solution (0.2 µg Cd and 0.5 µCi ^{109}Cd per ml of 10 mM Tris-HCl buffer, pH 7.4) was added to a 200 µl aliquot of the supernatant and the mixture was incubated (20 min; room temperature). Finally, 100 µl of 2% hemoglobin was added. Then the sample was heated (100°C; 2 min) and centrifuged

(10,000 x g; 5 min). After this step was repeated, the concentration of metallothionein was calculated using ^{109}Cd measurements (Beckman Gamma 4000) of supernatants from the sample and appropriate controls.

Metallothionein mRNA

Total RNA was extracted from hepatocytes using the method of Chomczynski and Sacchi (1987). Cells from 3-5 dishes were homogenized in 1 ml of guanidinium isothiocyanate, protein was removed using a phenol:chloroform:isoamyl alcohol mixture and RNA was precipitated with ethanol. RNA was dissolved in sterile, distilled, deionized water and the concentration of each sample was calculated using A_{260}. Dot blot and Northern blot analyses were conducted as described (Blalock et al., 1988), except 60-mer oligonucleotide probes specific for metallothionein -1 and -2 genes and corresponding to bases 16-76 from the 5'terminus (Anderson et al., 1987) were used for hybridization. The probes were 5'-end labeled with [γ -^{32}P]ATP (DuPont/NEN) using T_4 polynucleotide kinase (Bethesda Research Laboratories) and purified by chromatography (Sephadex G-50, Sigma) prior to hybridization. The specific activity of each probe was routinely 3.0 μCi/pmol as measured by Cerenkov counting (Beckman LS 7500). A ^{32}P-labeled ß-actin probe was used to verify uniformity of hybridization.

For Northern blots, total RNA was electrophoresed in a 1.1% agarose gel and transferred to a nitrocellulose filter (BA85; Schleicher and Schuell). Dot blot analyses were used to quantitate metallothionein mRNA. After hybridization, the ^{32}P content of each dot was measured by liquid scintillation counting (Beckman LS 7500). Molecules of metallothionein mRNA per cell were calculated as previously described

(Blalock et al., 1988) using an RNA/DNA ratio of 4.0, 6.4 pg of DNA per cell, and 100% efficiency of hybridization.

δ-Aminolevulinic acid dehydratase

The activity of δ-aminolevulinic acid dehydratase was measured by the method of Gurba and colleagues (1972). Cells from 1-3 dishes were harvested in 1 ml of phosphate buffer (0.04 M NaCl in 0.1 M sodium phosphate buffer, pH 6.7), homogenized (Polytron with P10 generator), and centrifuged (10,000 x g; 4°C). Then 250 μl of supernatant was pre-incubated with 25 μl of phosphate buffer containing 8.75 μmoles ß-mercaptoethanol for 1 h at 37°C. After pre-incubation, 75 μl of phosphate buffer containing 7.5 μmoles of δ-aminolevulinic acid was added to initiate the reaction. The formation of porphobilinogen at 37°C was terminated after 90 min by adding 350 μl 0.1 M $HgCl_2$ in 10% trichloroacetic acid. Porphobilinogen formation was linear for at least 2 h. Following centrifugation (100 x g; 5 min; 4°C), 500 μl of supernatant was added to 500 ul of Ehrlich's reagent (0.13 M dimethylaminobenzaldehyde and 4 M $HClO_4$ in glacial acetic acid). A_{555} was determined 30 min after color development was initiated and porphobilinogen concentration was calculated by comparison to standards. The specific activity of the enzyme is defined as nmoles porphobilinogen/min/mg protein.

Malondialdehyde

Malondialdehyde concentrations were measured by the thiobarbituric acid method as described by Buege and Aust (1978). Cells from 1-3 dishes were harvested in 2 ml of TCA-TBA-HCl reagent (15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid, 0.25 N HCl), and

heated (100°C; 15 min). After cooling, the flocculent precipitate was removed by centrifugation (1000 x g; 10 min). A_{535} was determined and the malondialdehyde concentration of the sample was calculated using $\epsilon_{535} = 1.56 \times 10^5\ M^{-1}cm^{-1}$.

Protein

Protein concentrations were determined by the method of Lowry and coworkers (1951). First, 1.0 ml of Lowry Reagent (0.58 mM $Na_2CuEDTA$, 0.18 M Na_2CO_3, 1% w/v sodium dodecylsulfate, and 0.1 M NaOH) was added to a sample or standard which was previously diluted to 0.1 ml. After the sample was mixed and incubated for 10 min, color development was initiated by adding 0.1 ml of Phenol reagent (Sigma). A_{500} was measured 30 min later and protein concentration was determined by comparison to a set of BSA standards.

Lactate dehydrogenase

Lactate dehydrogenase activity leaked from hepatocytes into culture medium was measured spectrophotometrically as the increase in NADH concentration from the oxidation of lactate (Amador et al, 1963). Briefly, 50 μl of culture medium was added to 1 ml of lactate dehydrogenase reagent (50 mmol lactate, 7 mmol NAD, pH 8.9) and A_{340} was monitored for several minutes. Lactate dehydrogenase activity was calculated as a rate using $\epsilon_{340} = 6.22\ mM^{-1}cm^{-1}$.

Statistical analyses

Data were subjected to analysis of variance and Duncan's multiple range test (SAS Institute, 1985).

RESULTS

Zinc Deficiency Study

Effect of Extracellular Zinc and Zinc-binding Ligands on Cellular Zinc Concentration

The zinc concentration in freshly isolated hepatocytes was similar to that in whole liver (Table 1). Hepatocytes were cultured in medium containing either 1, 16, or 48 μM zinc to assess the effects of extracellular zinc on cellular zinc concentration (Figure 1). Zinc-deficient (1 μM zinc) medium had no effect on the zinc concentration in hepatocytes during the 3 h attachment period and for up to 48 h of continuous culture. In contrast, zinc concentrations in hepatocytes cultured after the attachment period in medium containing either 16 or 48 μM zinc were significantly ($P \leq 0.05$) increased to 139 and 152% of the initial value, respectively, by 12 h. Zinc levels in these hepatocytes remained elevated at 48 h of culture.

To attempt to deplete hepatocyte zinc, the zinc concentration in the deficient medium was reduced to 0.3 μM by extracting zinc with a chelating resin (Table 4). Similar to the results presented in Figure 1, zinc deficient medium (0.3 μM zinc) did not reduce the zinc concentration of hepatocytes whereas medium containing 16 or 48 μM zinc increased cellular zinc (Figure 2).

The zinc-binding ligands, BSA and EDTA, were added to culture medium to attempt to reduce the availability of medium zinc and depress

Table 1. Zinc concentrations of rat liver and freshly isolated rat hepatocytes.

Tissue	n	Tissue zinc[#]
		nmoles Zn/mg protein
liver	4	1.5 ± 0.1
hepatocytes	19	1.7 ± 0.1

[#]Values represent the mean ± SEM.

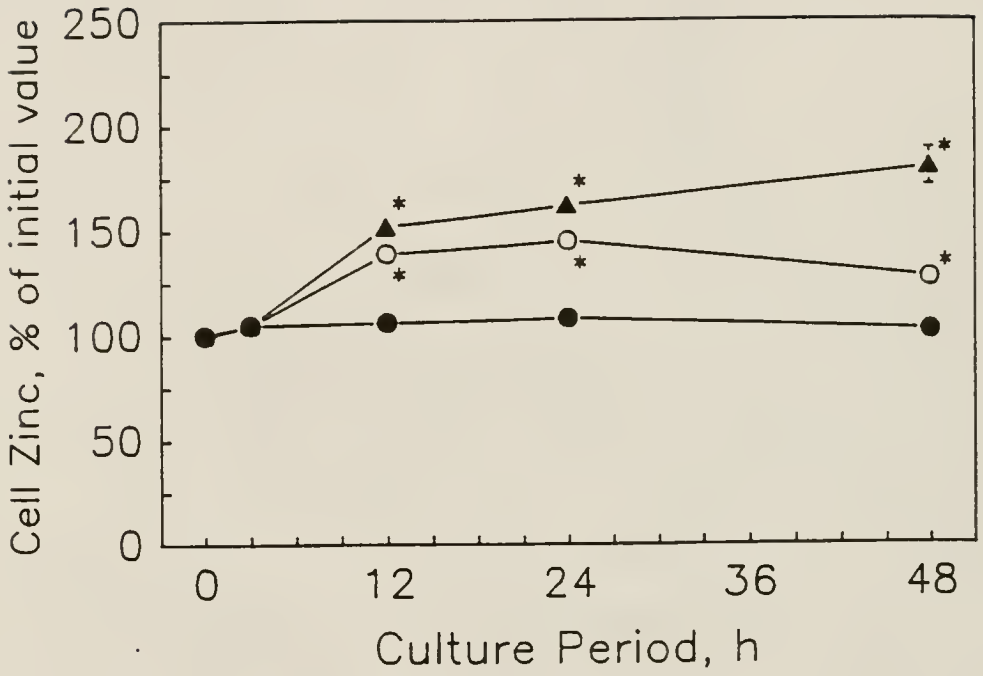

Figure 1. Effect of medium zinc concentration on zinc concentration in rat hepatocytes. Hepatocytes were cultured in Waymouth's medium containing BSA (2 mg/ml) and zinc at concentrations of either (•) 1, (○) 16, or (▲) 48 μM. At various times, hepatocytes were harvested and the cell zinc content was measured. Each point represents the mean ± SEM ($n \geq 7$ from at least two experiments) expressed as a percent of the initial value in fresh cells. *Significantly different ($P \leq 0.05$) from the initial value.

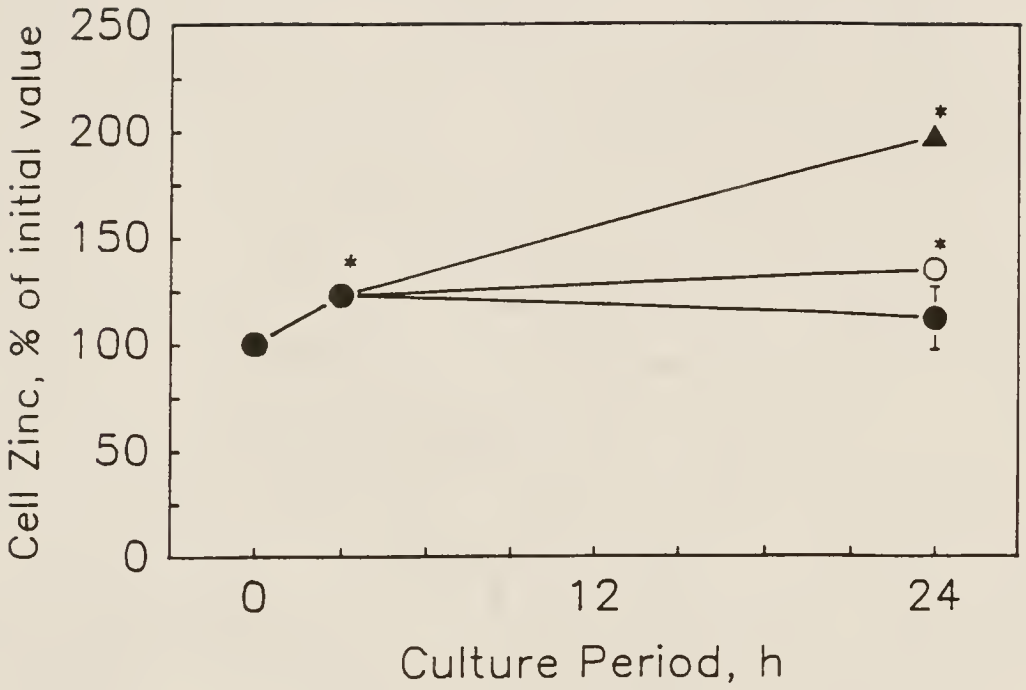

Figure 2. Effect of medium zinc concentration on zinc concentration in rat hepatocytes. Hepatocytes were cultured in Waymouth's medium containing BSA (2 mg/ml) and zinc at concentrations of either (•) 0.3, (○) 16, or (▲) 48 μM. At various times, hepatocytes were harvested and the cell zinc content was measured. Each point represents the mean ± SEM (n=4) expressed as a percent of the initial value in fresh cells. *Significantly different ($P \leq 0.05$) from the initial value.

cellular zinc concentration (Figure 3). BSA (Figure 3A) was chosen because it is the principal plasma zinc-binding protein and produces saturable zinc-uptake kinetics in hepatocytes (Pattison and Cousins, 1986). EDTA (Figure 3B) was selected because it has been shown to limit the availability of divalent cations to B and T lymphocytes (Zanzonico et al., 1981). BSA and EDTA each acted in a concentration-dependent fashion to reduce the increases in cellular zinc concentration produced by medium containing 16 or 48 μM zinc. However, neither BSA nor EDTA, at concentrations as high as 40 mg/ml and 98 μM, respectively, effected the level of zinc in hepatocytes cultured in medium with 1 μM zinc. For all subsequent experiments EDTA was not included in medium and BSA was included at 2 mg/ml, the standard concentration recommended for hepatocyte culture (Failla and Cousins, 1978b).

Effect of Extracellular Zinc Concentration on δ-Aminolevulinic Acid Dehydratase Activity

Since zinc dissociates relatively easily from the thiol groups of δ-aminolevulinic acid dehydratase (δ-ALA-D) (Tsukamoto et al., 1979), the effects of medium zinc on the activity of this zinc metalloenzyme were examined (Figure 4). In hepatocytes cultured in zinc deficient medium, δ-ALA-D activity was significantly ($P \leq 0.05$) reduced to 75% of the initial value by 3 h where it was maintained for up to 24 h. In comparison, in hepatocytes cultured after the 3 h attachment period with medium containing either 16 or 48 μM zinc, δ-ALA-D activity was restored to about 95% of the initial value by 24 h. Beyond 24 h, δ-ALA-D activity decreased regardless of medium zinc concentration.

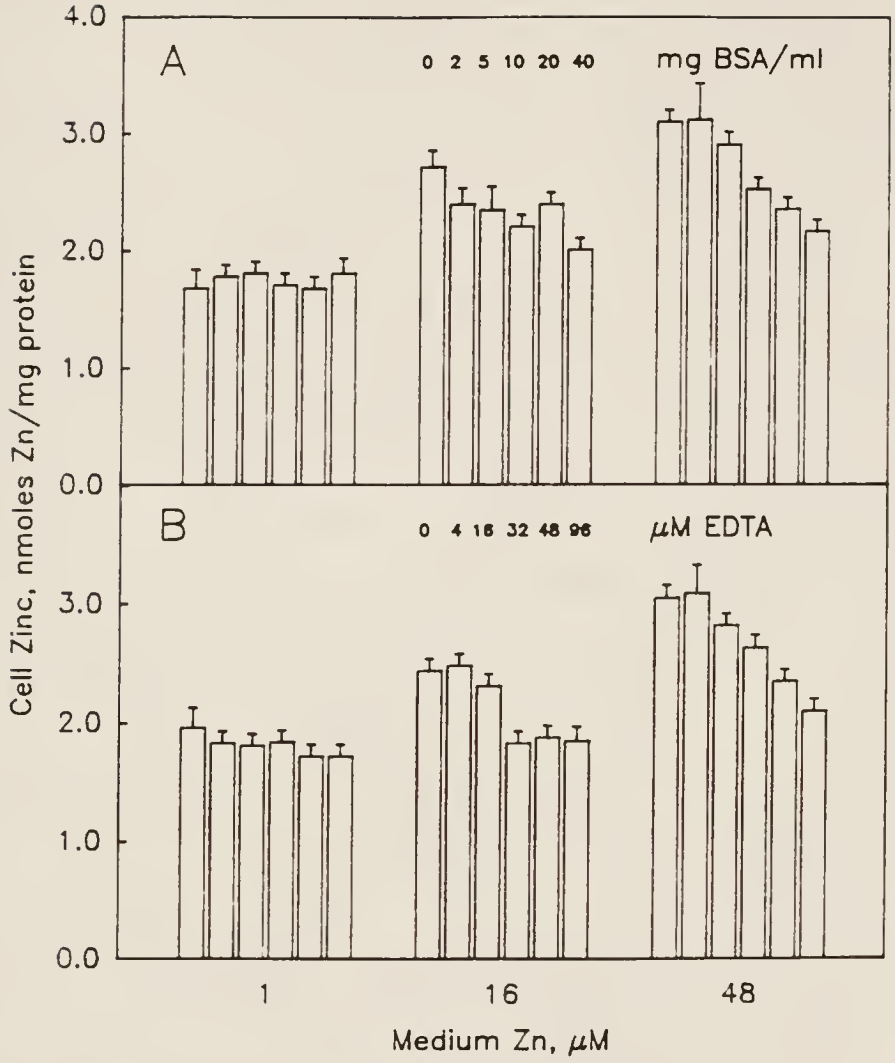

Figure 3. Effects of BSA and EDTA added to culture medium on zinc concentration in rat hepatocytes cultured with differing medium zinc concentrations. Three h hepatocyte cultures were incubated in Waymouth's medium containing zinc at concentrations of either 1, 16, or 48 μM. Media also contained BSA (A) or EDTA (B) at the concentrations indicated. After 21 h hepatocytes were harvested and the cell zinc content was measured. Each value represents the mean ± SEM (n=3).

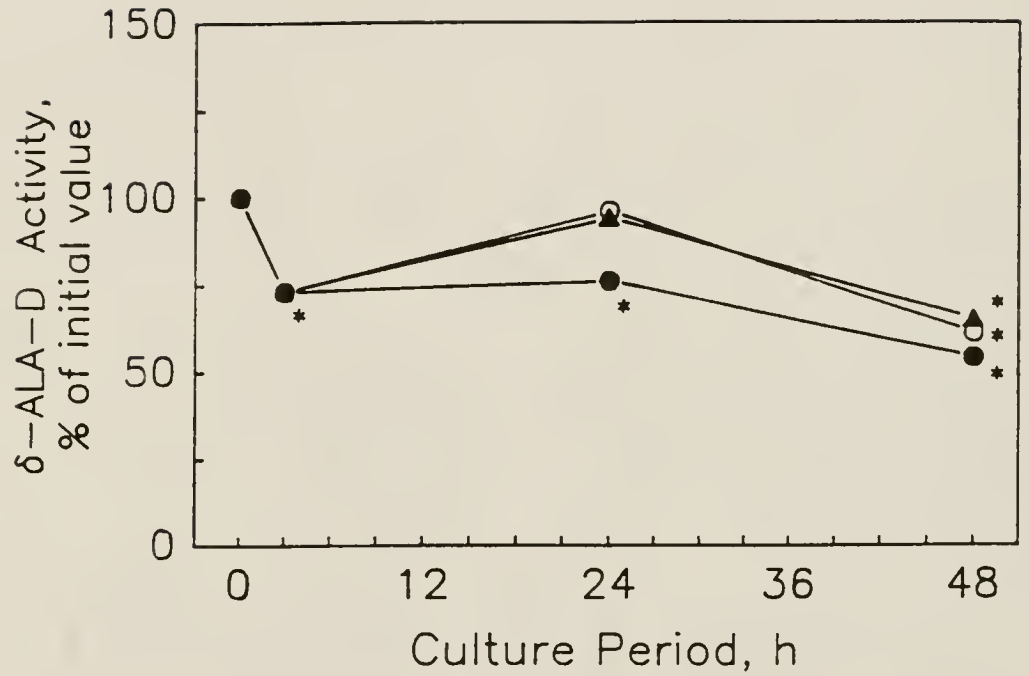

Figure 4. Reduction of δ-aminolevulinic acid dehydratase activity in rat hepatocytes as a function of medium zinc concentration. Hepatocytes were cultured in Waymouth's medium containing BSA (2 mg/ml) and zinc at concentrations of either (•) 1, (○) 16, or (▲) 48 μM. At various times, hepatocytes were harvested and δ-aminolevulinic acid dehydratase activity was measured. Each point represents the mean ± SEM (n≥4) expressed as a percent of the initial value in fresh cells. *Significantly different ($P \leq 0.05$) from the initial value.

Effect of Extracellular Zinc Concentration on Metallothionein Gene Expression

Levels of metallothionein mRNA and metallothionein protein as affected by medium zinc concentration are presented in Figure 5. Similar to cell zinc concentration, neither metallothionein mRNA (Figure 5A) nor metallothionein protein (Figure 5B) were affected by zinc deficient medium (1 μM zinc) during the 3 h attachment period and for up to 48 h of culture. However, in hepatocytes cultured in medium containing 16 or 48 μM zinc, both metallothionein mRNA and metallothionein protein were proportionately increased within 24 h and remained elevated at 48 h.

Effect of Extracellular Zinc Concentration on De Novo Synthesis of Hepatocyte Proteins

To assess the effects of medium zinc on synthesis of other proteins, hepatocyte polypeptides were labeled with ^{35}S-methionine and analyzed by two-dimensional polyacrylamide gel electrophoresis (Figure 6). Medium zinc concentration had no effect on Coomassie Blue stained polypeptides or ^{35}S-labeled polypeptides. Therefore, de novo synthesis of hepatocyte proteins other than metallothionein was not affected by varying levels of extracellular zinc.

Effect of Extracellular Zinc Concentration on Membrane Integrity

Membrane integrity was assessed by measuring LDH activity leaked from hepatocytes into medium after culture with medium containing various zinc concentrations (Table 2). Clearly medium zinc concentation had no effect on the leakage of this cytosolic enzyme from hepatocytes after either 24 or 48 h of culture.

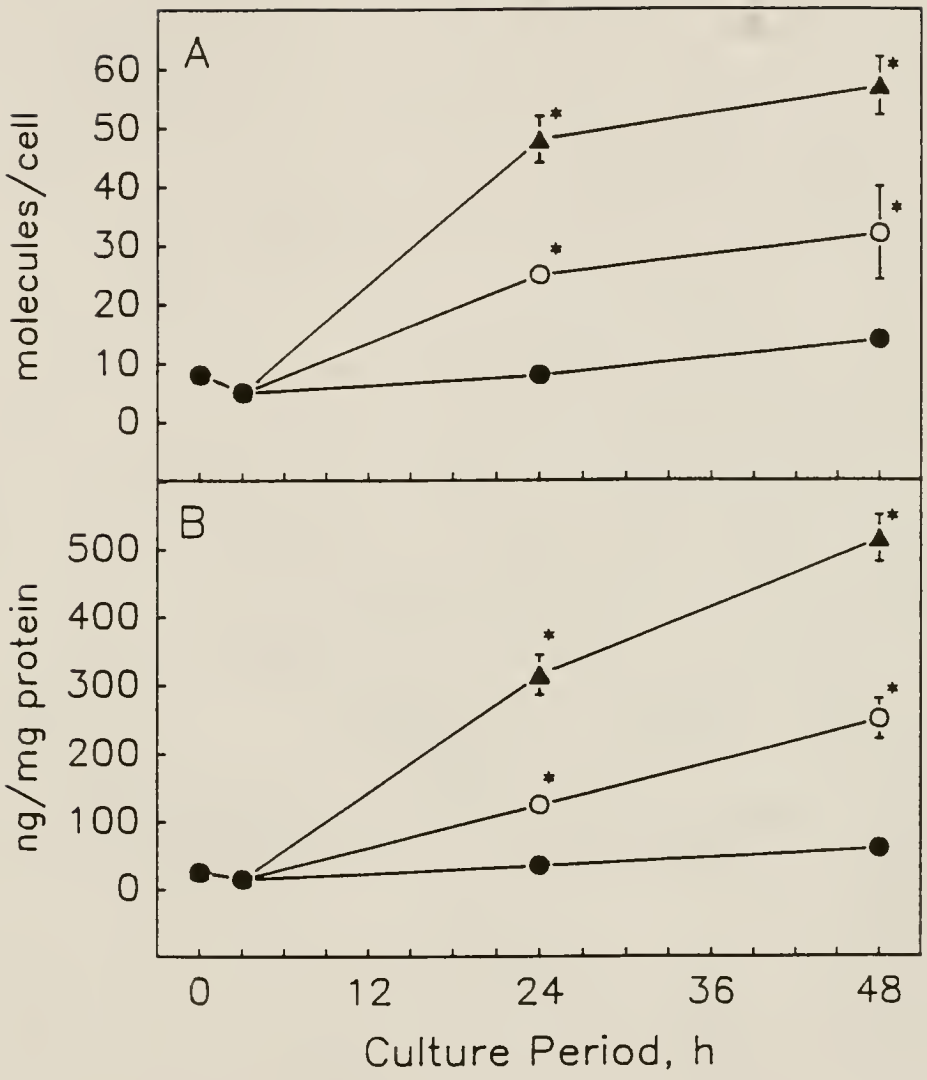

Figure 5. Effect of medium zinc concentration on metallothionein gene expression in rat hepatocytes. Hepatocytes were cultured in Waymouth's medium containing BSA (2 mg/ml) and zinc at concentrations of either (•) 1, (○) 16, or (▲) 48 μM zinc. At various times, hepatocytes were harvested and either metallothionein mRNA (A) or metallothionein protein (B) was measured. Each point represents the mean ± SEM (n=3 for A; n=7 for B).

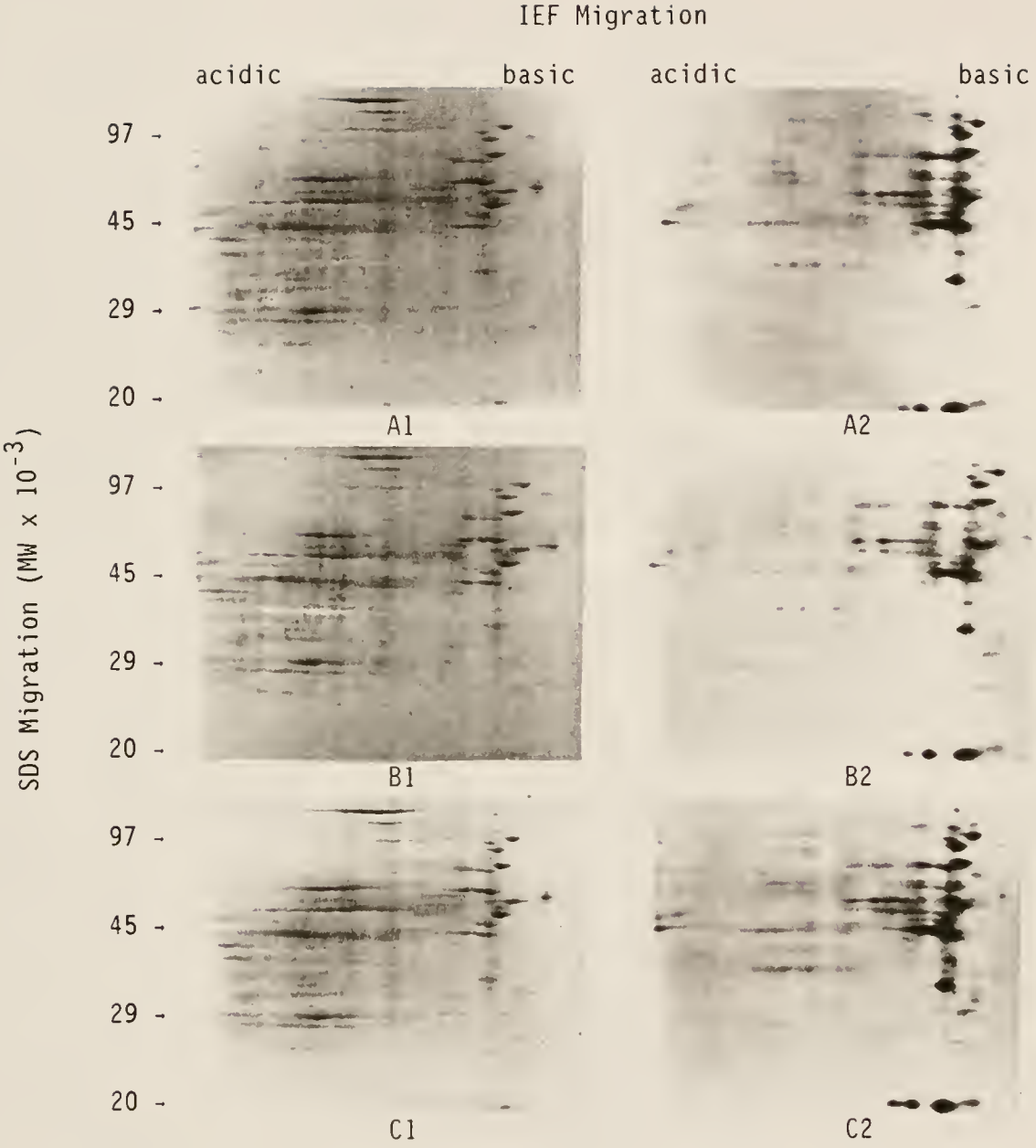

Figure 6. Effect of medium zinc concentration on de novo synthesis of hepatocyte proteins. Hepatocytes cultured for 18 h with BSA-supplemented Waymouth's medium containing 1 (A), 16 (B), or 48 (C) μM zinc were cultured for an additional 3 h with methionine-free medium containing the same levels of zinc and ^{35}S-methionine. Then hepatocytes were harvested and polypeptides were two dimensionally analyzed using isoelectric focusing (first dimension) and SDS polyacrylamide gel electrophoresis (second dimension). The Coomassie Blue stained polypeptides (1) and autoradiograms of ^{35}S-labeled polypeptides (2) are shown.

Table 2. Effect of medium zinc concentration on leakage of lactate dehydrogenase activity from rat hepatocytes into culture medium.

Culture Period	Medium Zn	LDH activity
h	μM	U/mg protein·h
3-24	1	10.5 ± 0.1
	16	9.8 ± 0.6
	48	10.3 ± 1.7
24-48	1	10.3 ± 0.7
	16	10.9 ± 1.1
	48	10.9 ± 0.2

Hepatocyte cultures were incubated in Waymouth's medium containing BSA (2 mg/ml) and zinc at the concentrations indicated. At the end of culture periods, lactate dehydrogenase activity leaked into the medium from hepatocytes was measured.

Effects of Extracellular Zinc Concentration on Zinc Efflux

Results of a study which used $^{65}Zn^{2+}$ as a tracer to examine the effects of medium zinc concentration on zinc efflux from hepatocytes are shown in Figure 7. Zinc efflux was reduced by medium containing 1 μM zinc compared to efflux from cells cultured with medium containing either 16 or 48 μM zinc.

Acute Phase Zinc Metabolism Study

Interleukin 6 Increases Metallothionein Gene Expression

To determine the effects of interleukin 1α and interleukin 6 on metallothionein gene expression, hepatocytes were cultured for 24 h in Waymouth's medium supplemented with various concentrations of the two cytokines (Figure 8). The medium was also supplemented with 1 μM dexamethasone since glucocorticoid hormones are often required for cytokine effects (Baumann et al., 1984; Koj et al., 1984). Incubation of hepatocytes with interleukin 6 led to concentration-dependent increases in metallothionein mRNA (Figure 8A). A maximal increase in metallothionein mRNA, approximately three times that of control, was achieved with an interleukin 6 concentration of 10 HSF units/ml (10 ng/ml). In contrast, interleukin 1α at concentrations as high as 1000 LAF units/ml (20 ng/ml) had no effect on metallothionein mRNA. The effects of these cytokines on metallothionein protein 24 h after addition to the culture medium were also examined (Figure 8B). Similar to its effect on the mRNA, interleukin 6 increased metallothionein protein levels in a concentration-dependent manner. Again, a maximal increase of approximately 3.5 times that of control cells was achieved with interleukin 6 at 10 HSF units/ml (10 ng/ml). In contrast,

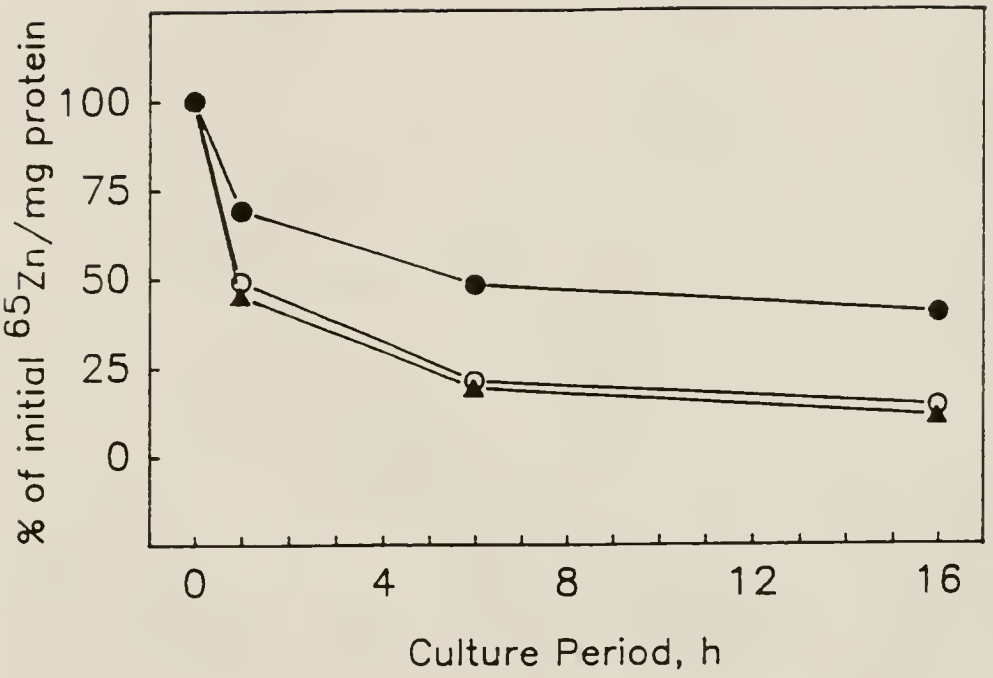

Figure 7. Zinc efflux from rat hepatocytes as a function of medium zinc concentration. Hepatocytes, labeled with ^{65}Zn for three h during the attachment period, were cultured in Waymouth's medium containing BSA (2 mg/ml) and zinc at concentrations of either (•) 1, (○) 16, or (▲) 48 μM. At various times, hepatocytes were harvested and ^{65}Zn content was measured. Each point represents the mean ± SEM (n=3).

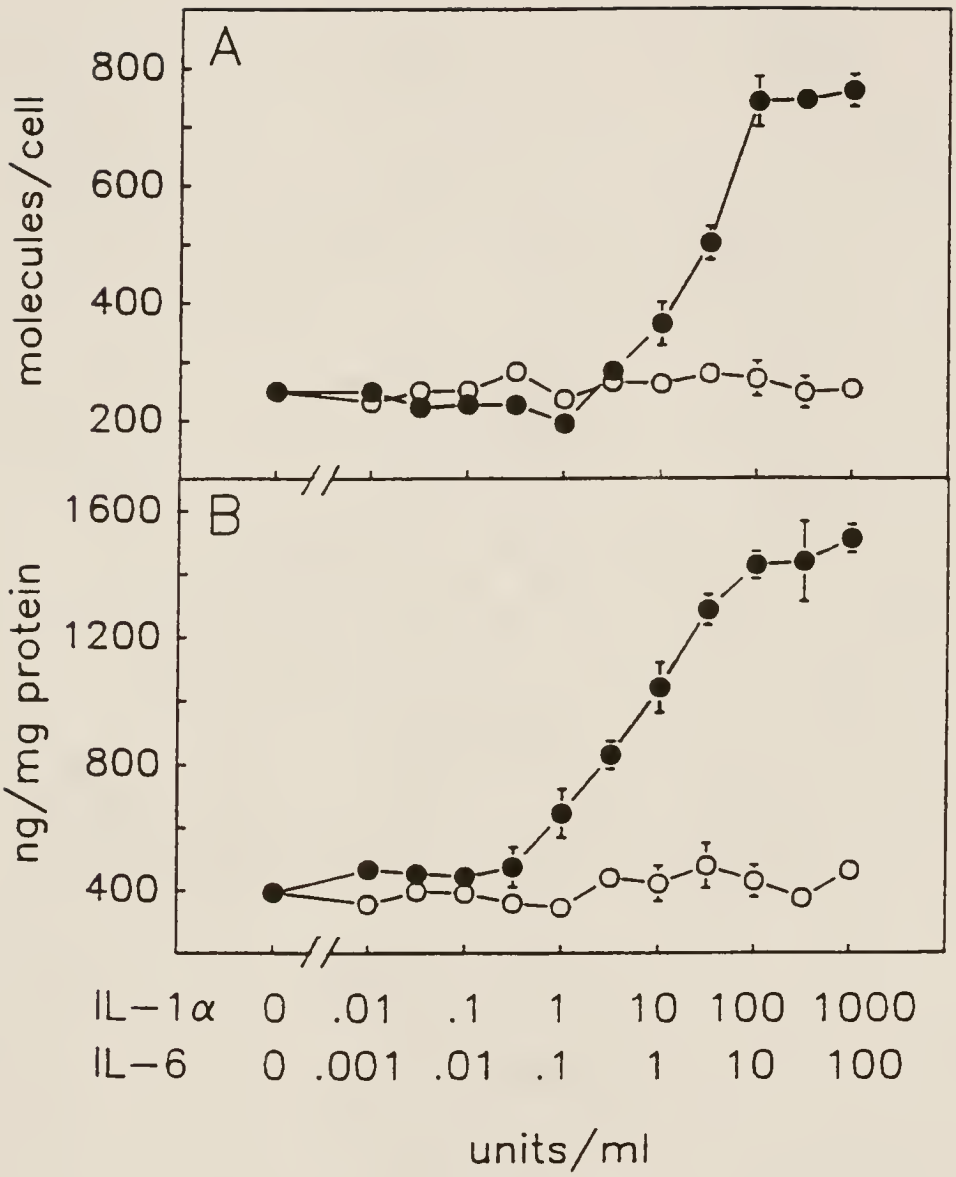

Figure 8. Dependence of rat hepatocyte metallothionein mRNA and metallothionein protein induction on cytokine concentration. Twenty-four h hepatocyte cultures were incubated with Waymouth's medium containing BSA (2 mg/ml), 1 μM zinc, 1 μM dexamethasone, and either (○) interleukin-1α or (●) interleukin 6 at the concentrations indicated. After 24 h the hepatocytes were harvested and either metallothionein mRNA (A) or metallothionein protein (B) was measured. Each point represents the mean ± SEM (n=4)

increasing amounts of interleukin 1α had no effect on metallothionein concentrations.

Hepatocytes were cultured for up to 48 h with either Waymouth's medium alone, medium supplemented with dexamethasone, or medium supplemented with dexamethasone and interleukin 6 at 10 HSF units/ml (10 ng/ml) to determine the temporal effects of interleukin 6 on metallothionein expression (Figure 9). Cultures with interleukin 6 exhibited time-dependent increases in both metallothionein-1 (Figure 9A) and metallothionein-2 (Figure 9B) mRNA. Interleukin 6 up regulated expression such that mRNA levels for both metallothionein-1 and -2 were increased over those in both control and dexamethasone-treated hepatocytes within 3 h of culture. The maximal increases in each mRNA were achieved after 12-18 h of culture. In addition, induction of metallothionein-2 mRNA reached levels approximately three times that of metallothionein-1 mRNA. Specificity of the individual oligonucleotide probes is shown by adding the values at 24 h, 150 and 600 molecules per cell, respectively. The sum agrees with the value of 750 molecules per cell for the combined probe shown in the concentration-response experiment (Figure 8A). Time-dependent increases in metallothionein protein levels were also produced by interleukin 6 (Figure 9C). Metallothionein was increased within 3 h of culture but did not reach maximal levels until approximately 36 h.

Extracellular Zinc and Glucocorticoid Hormone Affect Interleukin 6-Induced Metallothionein Expression and Cell Zinc Concentration

Zinc metabolism can be modulated via regulation of metallothionein by glucocorticoids (Failla and Cousins, 1978a; 1978b; Etzel et al.,

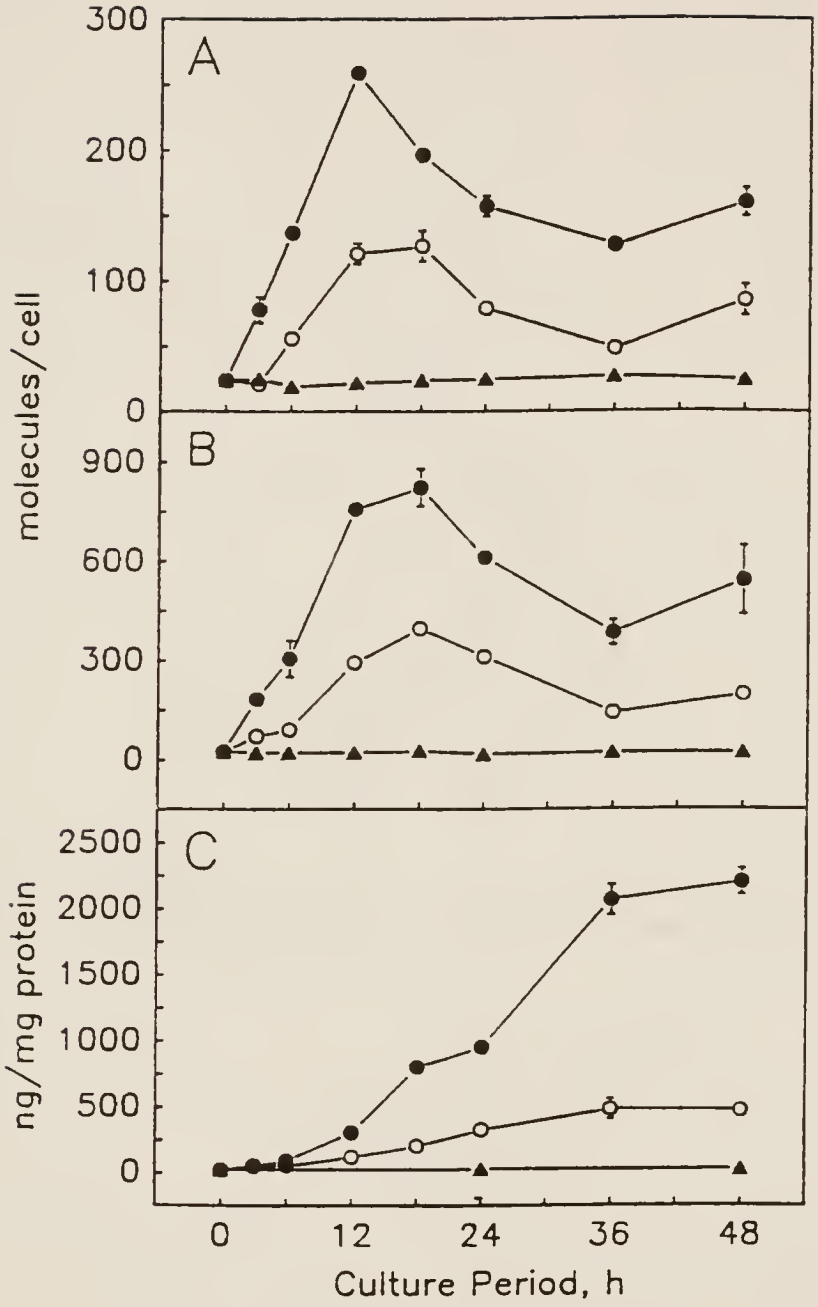

Figure 9. Time course of metallothionein-1 and -2 mRNA and metallothionein protein induction in rat hepatocytes by interleukin 6. Twenty-four h hepatocyte cultures were incubated for up to 48 h with either (▲) Waymouth's medium, (○) medium containing 1 μM dexamethasone, or (●) medium containing 1 μM dexamethasone and 10 HSF U interleukin 6/ml. All media contained BSA (2 mg/ml) and 1 μM zinc. At various times, hepatocytes were harvested and either metallothionein-1 mRNA (A), metallothionein-2 mRNA (B), or metallothionein protein (C) was measured. Each point represents the mean ± SEM (n=4).

1979) and dietary zinc (Blalock et al., 1988). To examine the extent to which extracellular zinc and glucocorticoid hormone affect interleukin 6-induced metallothionein expression, hepatocytes were harvested after 24 h of culture with various treatment combinations and RNA was extracted for evaluation by Northern blot analysis (Figure 10). A control experiment using a ß-actin DNA sequence showed uniform abundance of ß-actin mRNA (Figure 15). Therefore, changes in the intensity of the 550 base band representing metallothionein mRNA correspond to differences in treatments. Abundance of metallothionein mRNA was increased by the addition of zinc to the culture medium. At both 1 μM and 16μM zinc, the addition of interleukin 6 alone had little or no effect, whereas dexamethasone (1 μM) increased the metallothionein mRNA level dramatically. However, when interleukin 6 was added (10 HSF U/ml) with dexamethasone, levels were increased above the corresponding dexamethasone control cultures. Thus, metallothionein mRNA was most abundant in hepatocytes cultured with a combination of added zinc, dexamethasone, and interleukin 6.

The trends in metallothionein mRNA observed in the Northern blot were confirmed when quantitated by Dot blot analysis (Table 3). Levels increased 8.2 and 11.2 fold in response to interleukin 6 and dexamethasone in hepatocytes cultured with 1 and 16 μM zinc, respectively. Metallothionein protein levels and cellular zinc concentrations are also shown. Clearly, the same trends are reflected such that the highest levels of metallothionein and cell zinc were observed in hepatocytes cultured with a combination of added zinc, dexamethasone, and interleukin 6.

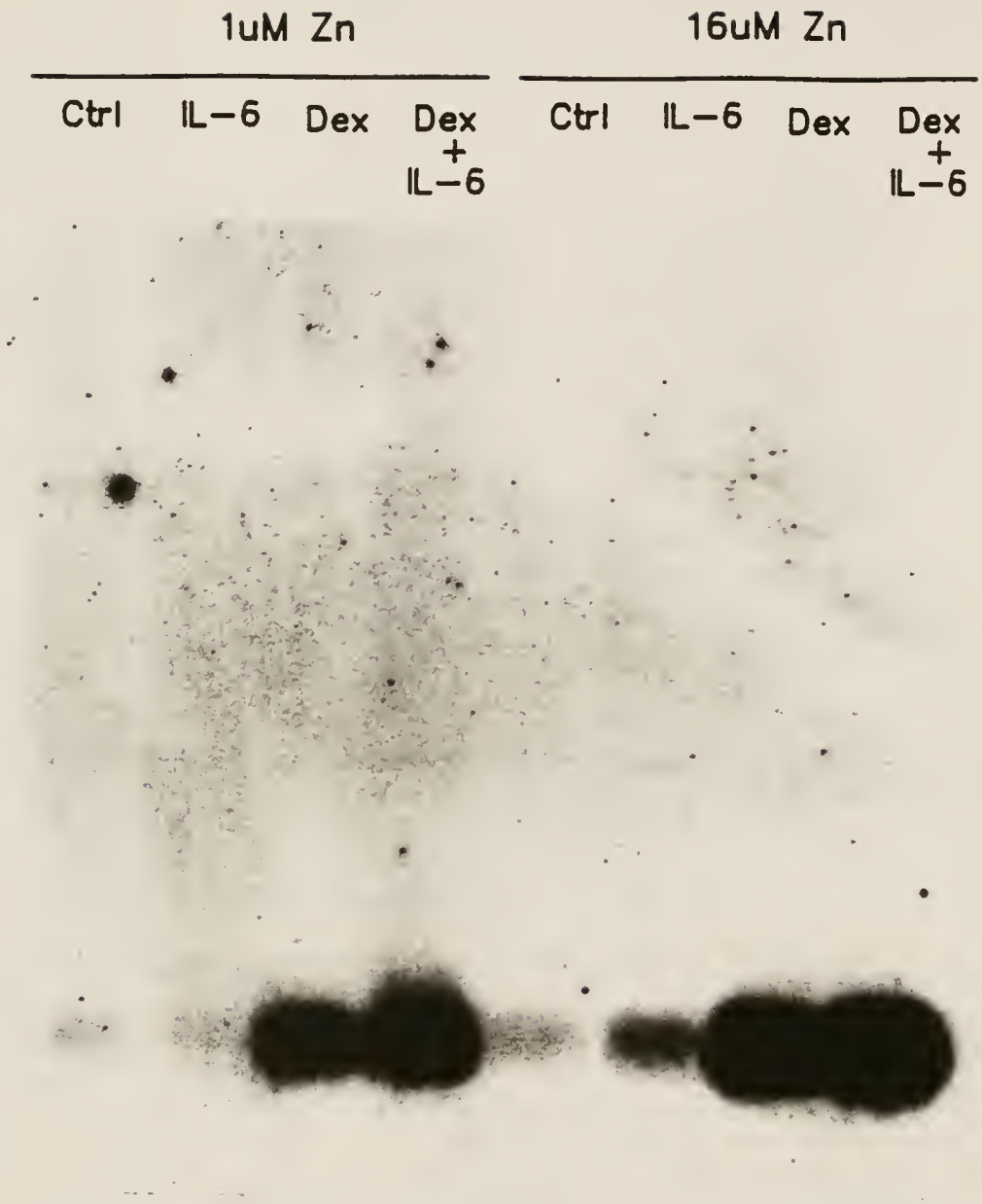

Figure 10. Northern blot illustrating the effects of combinations of zinc, dexamethasone, and interleukin 6 on metallothionein mRNA concentrations in rat hepatocytes. Twenty-four h hepatocyte cultures were incubated with Waymouth's medium containing BSA (2 mg/ml) and the treatment combinations indicated. Concentrations of dexamethasone and interleukin 6 were 1 μM and 10 HSF U/ml, respectively. After 24 h the hepatocytes were harvested and total RNA was evaluated by Northern blot analysis.

Table 3. Zinc and glucocorticoid dependence for interleukin 6 stimulation of metallothionein expression and cellular zinc accumulation.

Treatments	MTmRNA	MT	Cell Zn
	molecules/ cell	ng/mg protein	nmoles/mg protein
1μM Zn	60[a]	66[a]	1.8[ab]
+ IL-6	57[a]	69[a]	1.7[a]
+ Dex	275[b]	305[a]	2.2[c]
+ Dex + IL-6	495[c]	1415[b]	2.6[d]
16μM Zn	115[ab]	179[a]	2.0[bc]
+ IL-6	115[ab]	202[a]	1.9[ab]
+ Dex	576[c]	933[b]	3.4[e]
+ Dex + IL-6	1289[d]	3964[c]	5.3[f]
Pooled SEM	54	196	0.1
n	3	3	4

Twenty-four h hepatocyte cultures were incubated in Waymouth's medium containing BSA (2 mg/ml) and the treatment combinations indicated. Concentrations of dexamethasone and interleukin 6 were 1 μM and 10 HSF U/ml, respectively. After 24 h the hepatocytes were harvested and metallothionein mRNA (MTmRNA), metallothionein (MT) protein, and cell zinc concentrations were measured. Each value represents the mean ± SEM (n=3 or 4). Values with differing superscript letters are significantly different ($P \leq 0.05$).

Interleukin 6, Glucocorticoid Hormone, and Zinc Affect Iron (II)-Nitrilotriacetic Acid and Tert-Butyl Hydroperoxide-induced Lipid Peroxidation

To determine whether inducers of metallothionein expression and zinc accumulation provide cytoprotection, hepatocytes cultured for 24 h with combinations of added zinc, dexamethasone, and interleukin 6 were subsequently cultured with cytotoxic compounds. Interleukin 6, dexamethasone, and zinc combinations significantly ($P \leq 0.05$) altered iron (II)-nitrilotriacetic acid and tert-butyl hydroperoxide-induced lipid peroxidation in hepatocytes (Figure 11). The main effect of each mediator is shown in Figure 12. The addition of interleukin 6 or 48 μM zinc significantly ($P \leq 0.05$) reduced iron (II)-induced lipid peroxidation whereas dexamethasone increased peroxidation. In comparison, only dexamethasone reduced tert-butyl hydroperoxide-induced peroxidation.

Cytoprotection From Carbon Tetrachloride Toxicity is Consistent With Dependence Upon Metallothionein Induction and Zinc Accumulation

Survival curves for hepatocytes cultured with combinations of interleukin 6, dexamethasone, and zinc and subsequently exposed to carbon tetrachloride are shown in Figure 13A. Hepatocytes pre-treated with low extracellular zinc (1 μM) were the most susceptible to carbon tetrachloride-induced damage with only 25% surviving the first 6 h of exposure. The addition of interleukin 6 alone had no effect while the addition of 48 μM zinc or dexamethasone provided partial protection, significantly improving survival to 65 and 89%, respectively. In contrast, full protection was provided by adding a combination of both interleukin 6 and dexamethasone without additional zinc.

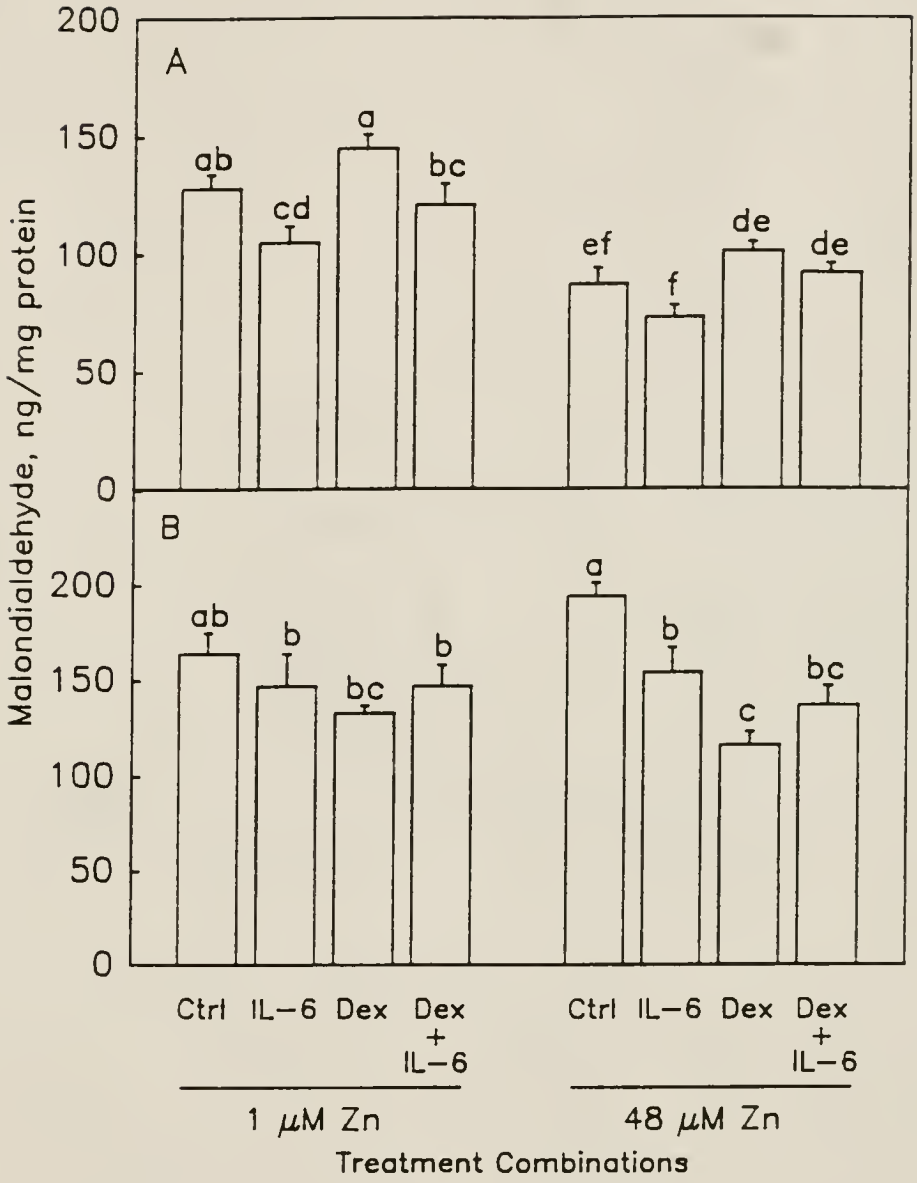

Figure 11. Cytoprotection against iron (II)-nitrilotriacetic acid and tert-butyl hydroperoxide-induced lipid peroxidation in rat hepatocytes. Hepatocytes were cultured for 24 h in Waymouth's medium containing BSA (2 mg/ml) and zinc, dexamethasone, and interleukin 6 in the combinations indicated. Concentrations of dexamethasone and interleukin 6 were 1 μM and 10 HSF U/ml, respectively. After pre-treatments, hepatocytes were cultured in BSA-supplemented Waymouth's medium containing iron (II)-nitrilotriacetic acid (A) or tert-butyl hydroperoxide (B) for 1 h. Each value represents the mean ± SEM (n=4). Values with differing letters are significantly different ($P \leq 0.05$).

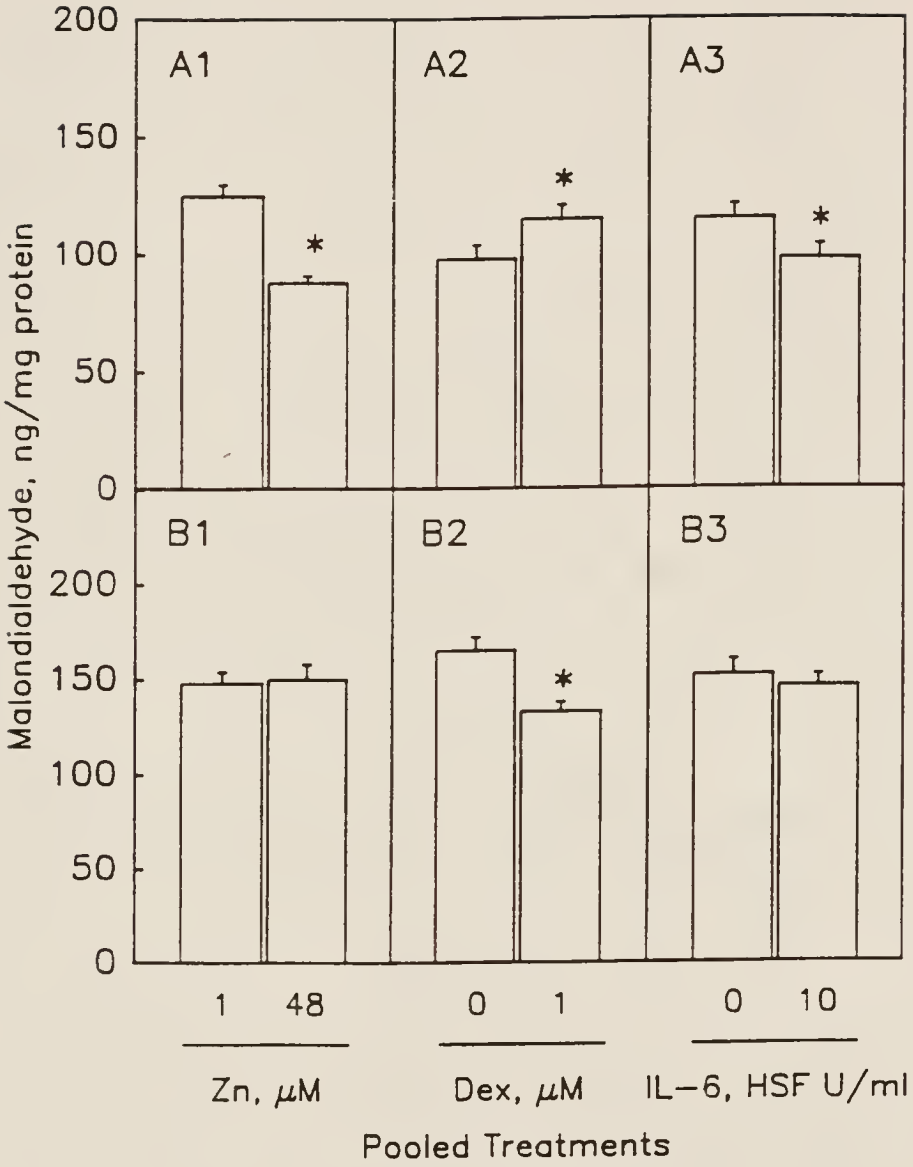

Figure 12. Cytoprotective effects of zinc, dexamethasone, and interleukin 6 against iron (II)-nitrilotriacetic acid and tert-butyl hydroperoxide-induced lipid peroxidation in rat hepatocytes. Data from Figure 11 were pooled to determine the individual cytoprotective effects of zinc (1), dexamethasone (2), and interleukin 6 (3) against iron (II)-nitrilotriacetic acid (A) and tert-butyl hydroperoxide (B) induced lipid peroxidation. Each value is the mean ± SEM (n=16). *Significantly different ($P \leq 0.05$).

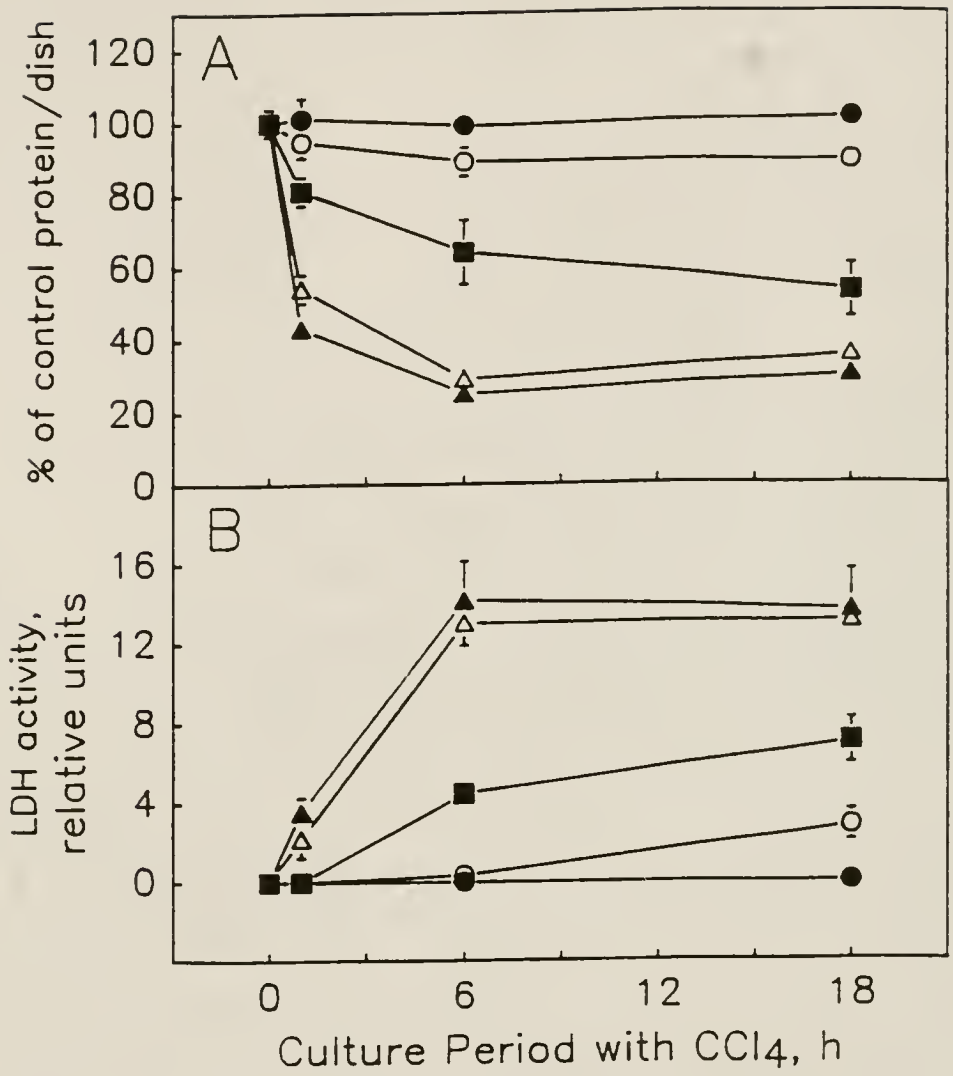

Figure 13. Cytoprotection against carbon tetrachloride toxicity in rat hepatocytes. Twenty-four h hepatocytes were treated for 24 h with BSA-supplemented (2 mg/ml) Waymouth's medium containing either (▲) 1 μM zinc, (△) 1 μM zinc and interleukin 6, (■) 48 μM zinc, (○) 1 μM zinc and dexamethasone, or (●) 1 μM zinc, dexamethasone, and interleukin 6. Concentrations of dexamethasone and interleukin 6 were 1 μM and 10 HSF U/ml, respectively. After pre-treatments, hepatocytes were cultured for up to 18 h in BSA-supplemented Waymouth's medium containing 5 mM CCl_4 and 140 mM DMSO or DMSO alone. Cell survival curves (A) were constructed by expressing the amount of cell protein remaining on dishes exposed to CCl_4 as a percent of that on control dishes. Cell leakage curves (B) were constructed using measurements of LDH activity leaked into the medium from hepatocytes exposed to CCl_4. Each point represents the mean ± SEM (n=4).

The trends observed in survival were reflected by the leakage of lactate dehydrogenase into the culture medium (Figure 13B). Hepatocytes pre-treated with low extracellular zinc (1 μM) exhibited the greatest leakage. The addition of interleukin 6 alone provided no protection while the addition of either 48 μM zinc or of dexamethasone reduced lactate dehydrogease leakage by 55 and 80%, respectively, over 18 h of exposure to carbon tetrachloride. Again, the addition of a combination of interleukin 6 and dexamethasone provided full protection, completely eliminating carbon tetrachloride-induced leakage over 18 h of exposure.

DISCUSSION

The purpose of the zinc deficiency study was to evaluate hepatocyte monolayer cultures as a cellular model of nutritional zinc deficiency. The approach taken was to examine a variety of indices of zinc status following culture of hepatocytes in medium containing zinc at levels of 1, 16, and 48 μM chosen to approximate the zinc concentrations in plasma of zinc deficient, normal, and supplemented animals. Plasma zinc levels equal to or exceeding 48 μM have been observed in zinc supplemented humans (Oelshlegel and Brewer, 1977) and are common in rats injected subcutaneously with zinc emulsions (Hill et al., 1984; Lee et al., 1989). Plasma zinc levels as low as 1 μM have never been achieved under practical dietary conditions. However, in severe nutritional zinc deficiency, plasma zinc concentrations are reduced in rats from the normal level of 16 to 24 μM to as low as 5 or 6 μM (Taylor et al., 1988).

The results of the study indicate that hepatocytes cultured as monolayers in zinc deficient medium maintain their zinc concentration (Figures 1 and 2) even when extremely high levels of the zinc-binding ligands EDTA and BSA are present in culture medium (Figure 3). This finding conflicts with the results of Guzelian and coworkers (1982) who conducted the only other study to examine the effects of zinc deficient medium on hepatocyte monolayers. Using similar conditions to those in the present study, the previous investigators observed a 60 to 70% loss

of cell-associated zinc within 24 h of culture. Considering the variety and importance of zinc's biological functions, it is debatable whether the hepatocyte could survive such a rapid and dramatic loss of zinc unless a sizeable portion of the zinc constituted a reserve.

To explain the disparity in results between the two cell studies, the possibility that the hepatocytes lost zinc during the isolation procedure was investigated. However, this was not the case, since zinc concentrations in whole livers were similar to those in freshly isolated hepatocytes (Table 1). The possibility that medium containing a lower level of zinc could deplete the zinc concentration of hepatocytes was also investigated by treating culture medium with a chelating resin to remove divalent cations (Table 4). After chelation, the calcium and magnesium levels were restored and the medium was used for hepatocyte culture. This zinc deficient medium containing 0.3 μM zinc also did not lower the cellular zinc concentration (Figure 2). If one assumes the level of zinc available to hepatocytes from the zinc deficient media used in the present study was less than or equal to that of zinc from zinc deficient plasma, hepatocyte monolayers reflect the ability of soft tissues such as liver to maintain their cellular zinc concentration even when severe signs of zinc deficiency are evident (Taylor et al., 1988).

The reduction in δ-aminolevulinic acid dehydratase (δ-ALA-D) activity in hepatocytes cultured in zinc deficient medium suggests the hepatocytes were mildly zinc deficient (Figure 4). The activity of this zinc metalloenzyme was reduced by 25% within 3 h of culture in zinc deficient medium, presumably reflecting a loss of zinc from essential thiol groups of the enzyme. This observation parallels that of Guzelian

and colleagues (1982) who found a 95% loss of δ-ALA-D activity within 24 h. δ-ALA-D activity is probably a good relative index of zinc deficiency up to the the first 24 h of culture since, in hepatocytes cultured with medium containing zinc at levels of 16 and 48 μM, activity was fully restored by this time (Figure 4). Beyond 24 h, however, use of δ-ALA-D activity as an index is confounded since activity decreases regardless of medium zinc concentration.

In normal liver, δ-ALA-D activity is one-to-two orders of magnitude greater than the activity of aminolevulinate synthase, the rate-limiting enzyme for heme biosynthesis (Meyer and Schmid, 1978). Therefore, the reduction in δ-ALA-D activity for hepatocytes cultured in zinc deficient medium may reflect a metabolic prioritization for zinc whereby zinc is lost first from a location of lesser immediate metabolic importance. The loss of zinc from δ-ALA-D and, perhaps, other low-priority cellular locations was not sufficient to significantly effect the overall cellular zinc concentration.

Different functions in other cultured mammalian cell-types may be more sensitive to zinc deprivation than δ-ALA-D activity in hepatocytes. For example, lymphocytes cultured in chelator-extracted medium containing 0.8 μM zinc had reduced T-killer activity (Flynn and Yen, 1981). Also, chelator-extracted medium containing serum and 2.2 μM zinc inhibited lymphocyte proliferation (Flynn, 1985). Cell proliferation was not examined in the present study because hepatocytes in primary culture do not replicate.

In studies in which intact animals were fed zinc deficient diets, hepatic metallothionein expression decreased significantly (Taylor et

al., 1988; Blalock et al., 1988), whereas in the present study neither metallothionein mRNA nor metallothionein protein levels in hepatocytes were affected by zinc deficient medium. This observation underscores that the zinc deficiency was mild. In comparison, cell zinc, metallothionein mRNA, and metallothionein were each increased in hepatocytes cultured with 16 or 48 μM zinc. These responses are similar to those observed in intact animals fed supplemental zinc (Blalock et al., 1988) or injected with zinc (Lee et al., 1989). The increases in zinc accumulation caused by media containing 16 and 48 μM zinc are linked to the increase in metallothionein expression in two ways. (i) The promoter regions of metallothionein genes contain metal-regulatory elements which are responsive to zinc (Hamer, 1986). Presumably, transcription is increased by zinc via a trans-acting, nuclear protein which binds zinc and interacts with the metal-regulatory element of the DNA. (ii) Zinc binds to and stabilizes apometallothionein. As a result, the protein's turnover is reduced (Dunn et al., 1987).

The medium containing 16 μM zinc was not expected to affect either cell zinc concentration or metallothionein expression since it was chosen to simulate the normal plasma zinc concentration. The zinc in the medium may have been more available than zinc in normal plasma since it was added in an ionic form as zinc sulfate. Nevertheless, sufficient BSA (2 mg/ml) was present to bind all of the zinc. Medium containing BSA produces saturable zinc uptake kinetics in hepatocytes which may reflect hepatic zinc uptake in vivo (Pattison and Cousins, 1986).

Varying the level of extracellular zinc had no effect on de novo synthesis of other hepatocyte proteins. This suggests that zinc-

responsive, metal-regulatory elements in the hepatocyte genome may be unique to metallothionein genes. In contrast, glucocorticoid-regulatory elements are ubiquitous since glucocorticoid hormones up-regulate the synthesis of not only metallothionein (Hamer, 1986) but also a broad spectrum of other hepatocyte proteins (Ivarie and O'Farrell, 1978).

Studies using intact animals and isolated cells have emphasized the critical role of zinc in maintaining membrane integrity (Bettger and O'Dell, 1981). In the zinc deficiency study, medium zinc concentration had no apparent effect on membrane integrity as judged by lactate dehydrogenase leakage. In comparison, previous work showed that hepatocytes cultured in medium containing 1 μM zinc were more susceptible to a variety of inducers of lipid peroxidation and free radical formation than those cultured in medium containing higher levels of zinc (Coppen et al., 1988). Extrapolating to zinc deficiency in intact animals, these observations suggest that in the absence of other complications hepatocyte membranes may function normally. However, in an oxidative environment characteristic of stress or infection, hepatocyte membranes and cell membranes of other soft tissues may be susceptible to damage. This view is consistent with Taylor et al. (1988). They found that the primary free radical defense system in the liver of severely zinc deficient rats was not seriously compromised but they cautioned that if free radical generation was increased, the defense system might be inadequate for overall protection.

The mechanism(s) by which soft tissues maintain cell-associated zinc despite severe depressions in extracellular zinc concentrations is not yet clear. Results of the present study indicate that maintenance

of liver zinc during zinc deficiency could be regulated, in part, by the hepatocyte itself. The kinetic experiment using $^{65}Zn^{2+}$ suggests that cell zinc concentrations may be maintained in hepatocytes cultured in zinc deficient medium by reducing zinc efflux (Figure 7). Presumably, the purpose of reduced efflux is to balance reduced zinc uptake (Pattison and Cousins, 1986). The application of computer modeling techniques to zinc kinetic and pool size data derived from hepatocytes could provide insights into the mechanism(s) involved in this process.

Substantial interest has been generated regarding the mechanisms that account for enhanced expression of acute-phase hepatic proteins in response to tissue injury, stress, and infection. Interleukin 1, a cytokine produced by activated macrophages and other cell-types, triggers a broad spectrum of systemic acute-phase responses in vivo including enhanced expression of hepatic acute-phase proteins and increased synthesis of other cytokines such as interleukin 6 (Dinarello, 1988). Administration of recombinant human interleukin 1α to rats induces the synthesis of hepatic metallothionein similar to other acute-phase proteins (Cousins and Leinart, 1988; Huber and Cousins, 1988). The increase in synthesis produces a tissue-specific redistribution of zinc with a transient depression of zinc in the plasma and concomitant uptake of zinc by the liver, bone marrow, and thymus. Similar changes are triggered by dibutyryl cAMP, endotoxin, and other mediators with interleukin 1-like activity (Cousins, 1985) and have been verified by simulation and modeling techniques (Dunn and Cousins, 1989). Results of the cytokine study examining acute-phase zinc metabolism in hepatocytes show that interleukin 6 rather than interleukin 1 is a mediator of

metallothionein production and changes in zinc metabolism at the level of the hepatocyte. Further, interleukin 6 induced changes provide cytoprotection from carbon tetrachloride-induced hepatotoxicity via a mode consistent with dependence upon increased cellular metallothionein.

Often the results of studies using cultures of various cell-types to assess the effects of cytokines on acute-phase protein synthesis seem to conflict. Differences in responsiveness to individual cytokines between studies are due to factors related to specific cell-types or lines or to differences in evaluation criteria such as measurement of a specific protein versus its mRNA (Morrone et al., 1988). In the present study, interleukin 1 had no effect on metallothionein mRNA in isolated hepatocytes (Figure 8) whereas Karen et al (1985) have shown that interleukin 1 increases metallothionein mRNA in Hep G2 hepatoma cells. The present results are consistent with those of others (Andus et al., 1988; Castell et al., 1988) who found that interleukin 6 affects the synthesis of a broad spectrum of acute-phase proteins in hepatocytes while interleukin 1 regulates only a few.

Interleukin 6 gave a maximal increase in metallothionein protein at 10 HSF units/ml (10 ng/ml). This concentration agrees well with the value of 30 HSF units/ml reported for maximal induction of other acute-phase proteins (Andus et al., 1988). The increase in metallothionein expression is probably dependent upon changes initiated at the level of transcription since metallothionein mRNA was maximally induced by the same level of interleukin 6.

Glucocorticoids may play an important role in regulating the acute-phase response. Both interleukin 1 and interleukin 6 stimulate the

release of corticotropin from cultured pituitary cells, suggesting that these cytokines increase glucocorticoid levels in vivo (Woloski et al., 1985). Glucocorticoids stimulate metallothionein synthesis in hepatocytes both in vivo (Etzel et al., 1979) and in vitro (Failla and Cousins, 1978a; 1978b). This ability is due to glucocorticoid responsive elements in the promoter region of the metallothionein genes (Hamer, 1986).

In the present study, glucocorticoid (dexamethasone) was required for interleukin 6 to up-regulate metallothionein synthesis. Glucocorticoid-dependency has also been recognized for interleukin 6 regulation of other acute-phase proteins (Baumann et al, 1984; Koj et al, 1984). Since interleukin 6 alone stimulates manganous superoxide dismutase gene expression in primary hepatocytes (with W. Dougall and H. Nick; Figure 16), glucocorticoid hormones are not required for synthesis of functional interleukin 6 receptors. A possible explanation for the glucocorticoid-dependency for interleukin 6 stimulation of expression of metallothionein and some other acute-phase proteins is that glucocorticoids bind to and alter the conformation of the promoter regions of the genes via their receptor proteins. Then interleukin 6 or a second messenger can act via their respective nuclear regulatory proteins. This glucocorticoid-dependency for interleukin 6 regulation in primary hepatocytes may reflect a need for a basal level of glucocorticoid which normally bathes the liver in vivo to facilitate expression of some liver functions. Alternatively, a possible effect of increased levels of circulating glucocorticoids on the liver during inflammation may be to shift the target tissues of interleukin 6 from

cell populations such as monocytes to other cells such as hepatocytes (Amrani et al., 1986; Bauer et al., 1989).

The ability of increased levels of extracellular zinc to facilitate interleukin 6-induced metallothionein production and cellular zinc accumulation (Table 3) can be attributed to two mechanisms. (i) Zinc binds to and stabilizes interleukin 6-induced apometallothionein so that the protein's turnover is reduced (Dunn et al., 1987). (ii) The promoter regions of the metallothionein genes contain metal regulatory elements that are responsive to zinc (Hamer, 1986).

If one assumes that each molecule of metallothionein binds seven atoms of zinc, the increases in cellular zinc in hepatocytes cultured with either added zinc or dexamethasone are accounted for by the increases in cellular metallothionein (Table 3). In comparison, the increases in cellular zinc in hepatocytes cultured with dexamethasone and interleukin 6 together are less than would be expected for the corresponding increases in metallothionein. Therefore, the addition of dexamethasone and interleukin 6 together may trigger a change in the intracellular distribution of zinc such that the portion of zinc not associated with metallothionein is reduced.

The reason for induction of hepatic metallothionein synthesis and zinc accumulation by acute-phase mediators is not yet clear. Chvapil and colleagues (1976; 1977) believe the purpose of decreasing plasma zinc is to increase the phagocytic activity of macrophages and the functional activities of other circulating cells. Another potential benefit of moving zinc out of the plasma and into organs such as the liver is to enhance zinc's availability to these tissues. Based on

zinc's role in stabilizing membranes (Bettger and O'Dell, 1981; Girotti et al., 1986) and metallothionein's purported role as a radical scavenger (Thornalley and Vasak, 1985), it has been proposed that zinc and/or metallothionein may play important intracellular roles as antioxidants by protecting hepatocytes and other cells during infection, stress, or tissue injury when host-generated cytotoxic oxygen species are produced in large quantities (Thomas et al., 1987; Coppen et al., 1988; Abel and Ruiter, 1989). In the present study, several cytotoxic compounds were screened for their abilities to increase lipid peroxidation or reduce cell survival (Table 5). Subsequently, these cytotoxic compounds were applied to hepatocyte cultures pre-treated with inducers of metallothionein synthesis and zinc accumulation to determine whether the inducers could provide cytoprotection.

The protective effects of interleukin 6 and zinc against iron (II)-induced lipid peroxidation and of dexamethasone against tert-butyl hydroperoxide-induced lipid peroxidation are probably not related to cellular metallothionein and zinc because they do not correlate well with metallothionein induction and zinc accumulation (Table 3). Zinc probably protects hepatocytes from iron (II)-induced lipid peroxidation by inhibiting iron uptake (Coppen et al., 1988) while interleukin 6 may protect the cells by inducing manganous superoxide dismutase synthesis (Figure 16). The inhibition of tert-butyl hydroperoxide-induced lipid peroxidation by dexamethasone could be due to a plethora of glucocorticoid effects in hepatocytes.

Only cytoprotection against carbon tetrachloride hepatotoxicity showed a strong correlation with levels of cellular metallothionein and

zinc. This result fits the theoretical framework of a functional role of zinc and/or metallothionein in membrane stabilization. Metallothionein can be envisioned as providing stabilization either directly as a radical scavenger or by binding $CCl_3^{\cdot}$ radicals or indirectly as a zinc donor to membrane sites or to specialized components such as the cytochrome P_{450} system. Zinc has been shown to inhibit this system by stabilizing NADPH (Chvapil et al., 1976; Ludwig et al., 1980; Jeffrey, 1983).

SUMMARY AND CONCLUSIONS

Hepatocytes cultured in zinc deficient medium maintained their zinc concentration similar to livers of zinc deficient animals despite an extracellular environment sufficiently zinc deficient to cause partial loss of zinc-dependent δ-aminolevulinic acid dehydratase activity. Therefore, hepatocyte monolayers may be a good cellular model to study the effects of zinc deficiency on the metabolism of livers and other soft tissues. Apparent membrane integrity, metallothionein expression, and de novo protein synthesis were unaffected by zinc deficient medium suggesting that in the absence of other complications such as tissue injury, stress, or infection, the defense system of the liver is adequate for overall protection.

Studies which utilized hepatocytes to examine acute-phase regulation revealed that, at the cellular level, interleukin 6, rather than interleukin 1, is a major physiological determinant of metallothionein expression and zinc metabolism. These effects of interleukin 6 require glucocorticoids and are optimized by increased levels of extracellular zinc. Since interleukin 1 does not elicit changes in metallothionein expression at the cellular level, measures of metallothionein mRNA, metallothionein protein, and cell zinc concentrations in hepatocytes may provide sensitive bioassays to functionally discriminate between interleukin 1 and interleukin 6 activity.

Interleukin 6 also induced changes which provided cytoprotection from carbon tetrachloride hepatotoxicity in a manner consistent with dependence upon increased cellular metallothionein and/or zinc. Since metallothionein is regulated similarly to other acute-phase proteins and may function in cytoprotection, it should be classified as an acute-phase protein. If metallothionein and/or zinc have important cytoprotective roles, a dietary zinc deficiency compounded by chronic infection, stress, or tissue injury could have an adverse effect on liver metabolism. Also, in conditions of chronic elevation of interleukin 6, the dietary zinc supply may be an important determinant in the physiological manifestations induced by this cytokine.

The findings of these studies, taken together with the results of other investigations, may provide a mechanism and reason to explain interleukin 1-triggered up-regulation of metallothionein and zinc accumulation in hepatocytes during the acute-phase response (Figure 14). Under normal conditions and in zinc deficiency, hepatic membrane stability is adequate. However, during tissue injury, stress, and infection when levels of host-generated cytotoxic oxygen species are high, hepatic membranes are at risk for damage, particularly in zinc deficient animals. During the acute-phase response, interleukin 1 is released from macrophages (Dinarello, 1988) and, in turn, stimulates the release of corticotropin which causes adrenal steroidogenesis (Woloski et al., 1985). Glucocorticoids can act on hepatocytes to increase metallothionein production as well as to feedback-inhibit the release of interleukin 1 from macrophages (Woloski et al., 1985). Interleukin 1 also stimulates the synthesis of interleukin 6 by some cell-types (Van

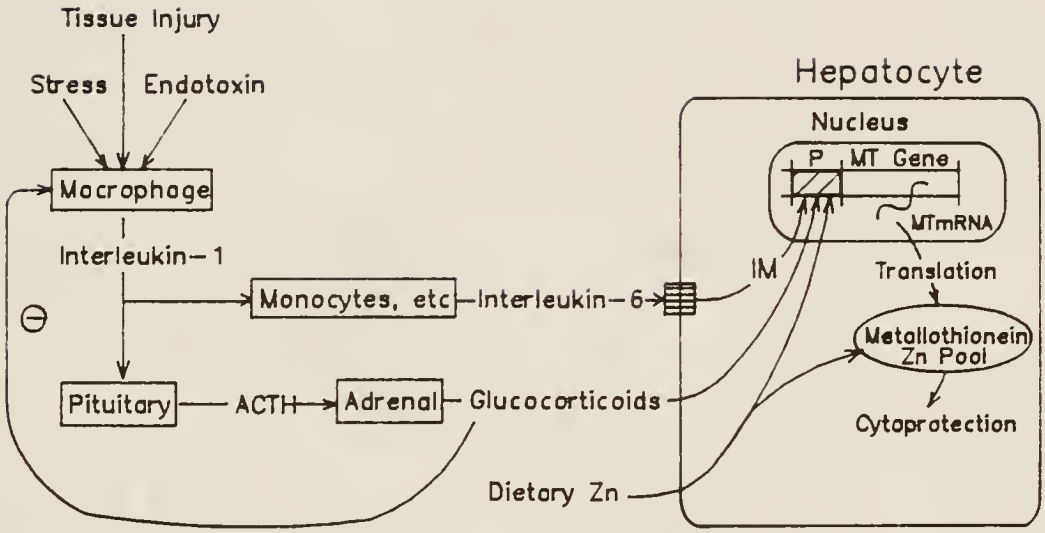

Figure 14. Interleukin 1-triggered up-regulation of metallothionein gene expression and zinc metabolism in hepatocytes.

Damme et al., 1987; Walther et al., 1988; Zhang et al., 1988). In the presence of glucocorticoids, interleukin 6 increases metallothionein production and zinc uptake by hepatocytes. These interleukin 6 effects are optimized by increased levels of extracellular zinc. The functional role of increased hepatic metallothionein production and zinc accumulation may be to provide cytoprotection.

APPENDIX

Table 4. Mineral composition of culture media and sera.

Medium or serum	Zinc	Magnesium	Calcium
	(μM)	-------(mM)-------	
Fetal bovine serum	45.0	---	---
Chelated fetal bovine serum[#]	5.0	---	---
Waymouth's medium	0.7	2.9	0.8
Chelated Waymouth's medium[#]	0.3	0.3	0.2
Repleted Waymouth's medium[*]	0.3	2.3	0.8

[#]Serum or medium was treated with Chelex-100 resin to remove divalent cations.

[*]Medium was treated with Chelex-100 resin and then magnesium and calcium were restored to their initial levels by adding $MgCl_2$ and $CaCl_2$, respectively.

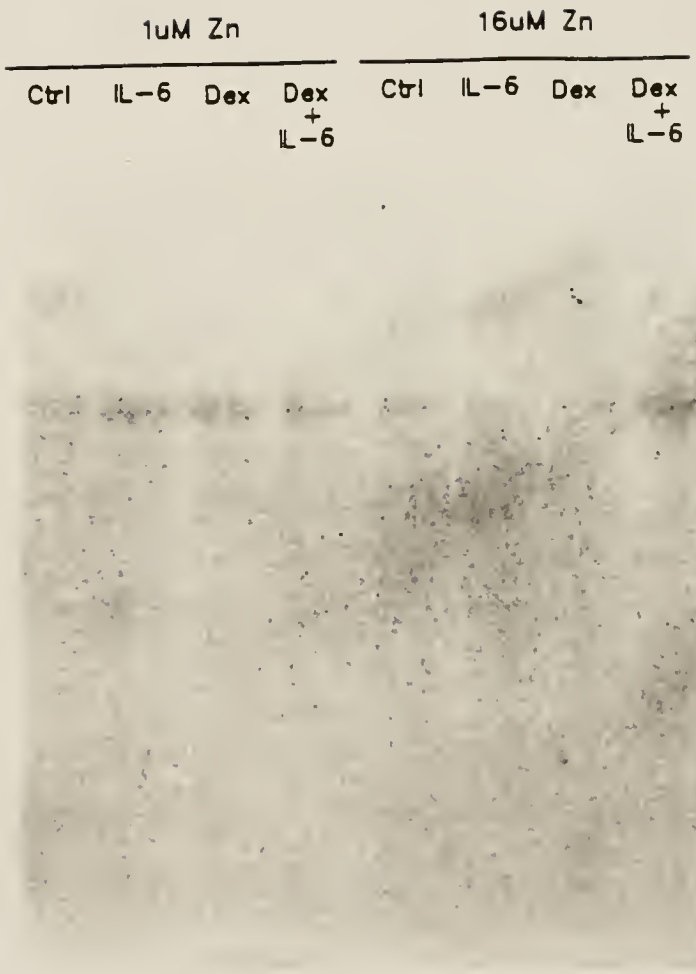

Figure 15. Northern blot illustrating the effects of combinations of zinc, dexamethasone, and interleukin 6 on ß-actin mRNA concentrations in rat hepatocytes. Twenty-four h hepatocyte cultures were incubated with Waymouth's medium containing BSA (2 mg/ml) and the treatment combinations indicated. Concentrations of dexamethasone and interleukin 6 were 1 μM and 10 HSF U/ml, respectively. After 24 h the hepatocytes were harvested and total RNA was evaluated by Northern blot analysis.

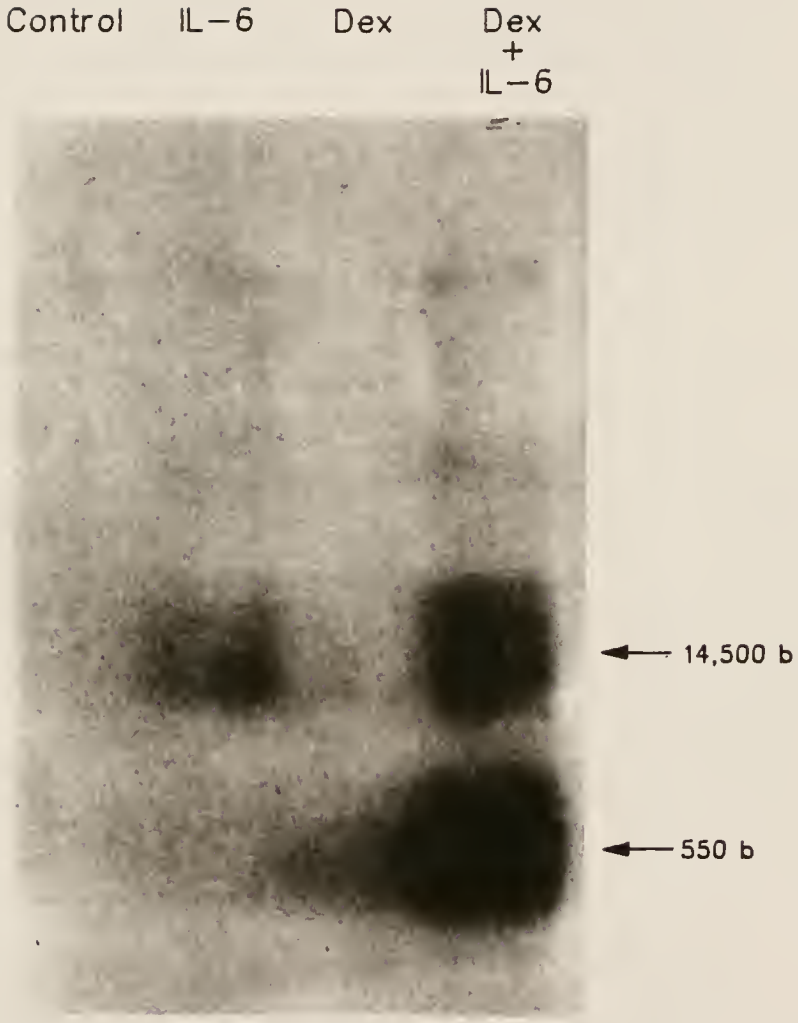

Figure 16. Northern blot illustrating the effects of combinations of dexamethasone and interleukin 6 on metallothionein and manganous superoxide dismutase mRNA concentrations in rat hepatocytes. Twenty-four h hepatocyte cultures were incubated with Waymouth's medium containing BSA (2 mg/ml), 1 μM zinc, and the treatment combinations indicated. Concentrations of dexamethasone and interleukin 6 were 1 μM and 10 HSF U/ml, respectively. After 24 h the hepatocytes were harvested and total RNA was evaluated by Northern blot analysis. The 14,500 and 550 nucleotide bands represent manganous superoxide dismutase and metallothionein mRNA's, respectively.

Table 5. Effects of cytotoxic compounds on cell survival and lipid peroxidation of rat hepatocytes.

Cytotoxic compound	Dose	N	Culture period (h to h)	Damage index[#]	% of control
iron(II)-nitrilotriacetic acid	100 μM	4	48-49	malondialdehyde	972*
				cell survival	102
tert-butylhydroperoxide	2.5 mM	4	48-49	malondialdehyde	809*
				cell survival	90
carbon tetrachloride	5.0 mM	4	48-49	cell survival	43*

[#]Cell survival was measured as cell protein/dish.

*Significantly different ($P \leq 0.05$) from control.

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BIOGRAPHICAL SKETCH

Joseph James Schroeder III, the eldest of Joe and Darlene Schroeder's four children, was born in Columbia, Missouri, on March 9, 1960. He received his early education at West Boulevard and Russell Boulevard Elementary Schools and West Junior High School. Joseph graduated with honors from Rock Bridge High School in 1978 and was awarded a Curator's Scholarship to attend the University of Missouri where he earned the B.S. degree in biochemistry in 1983. In 1985 Joseph married Miss Stephanie Lynn Zeek of Lake Ozark, Missouri, and shortly thereafter completed the M.S. degree in nutrition under Dr. Dennis T. Gordon. In the same year he was awarded a U.S.D.A. predoctoral fellowship to pursue the Ph.D. degree in nutrition under Dr. Robert J. Cousins at the University of Florida. He was conferred the degree in August 1990. Among other distinctions he achieved during his education, as a doctoral student Joseph received the American Institute of Nutrition 1989 Graduate Student Research Award for an outstanding paper of original research.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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